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The Journal of Biochemistry, Vol. 41, No. 1, 1954

SUCCESSIVE EXTRACTION OF TISSUE PROTEINS

I. LIVER, KIDNEY, AND LYMPH-NODE

By SHOJIRO NAKAMURA, YASUSHI HAYASHI, MOTOYUKI TAKAHASHI AND TOMOHARU ZAISEN

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(Received for publication, September 15, 1953)

For the study of tissue proteins, they must be extracted from tissues by means of a suitable solvent. As such solvent buffered or non-buffered solutions of sodium chloride, or potassium chloride of isotonic or other concentrations, and sometimes, non-electrolytes such as sucrose solutions are usually employed. Of the conditions of extraction, the pH, ionic strength, and kinds of solvent are taken into account, but little attention is paid to the quantity of the extracting solvent. As will be seen later, when the lymph-nodes were extracted scccessively with the physiological saline solution, the first and the third extracts were different regarding the proportion of the components extracted. Hence it seems necessary to examine the process of extraction.

As to the extraction of low molecular substances, especially of amino acids, recent investigations by Synge (*1*), Craig (*2*), and many others have made it possible to treat the process theoretically. For the extraction of proteins, however, the chromatographic techniques can hardly be applied, because of the lack of suitable stationary phases or protein solvents immiscible with water (*3*). In the present study, the tissue debris, or actually, precipitates (the debris including a little amount of supernatant solution) were regarded as a stationary phase. This makes the theoretical treatment insufficient but at least allows us to understand the extraction process closely.

THEORETICAL

For the case of the theoretical treatment, be the procedure of the successive extraction as follows: To a known quantity of an organ brei a proportional quantity of a suitable solvent is added and centrifuged. To the precipitate the same proportional quantity of the solvent is again added and centrifuged. And so on.

1. *Extraction rate*

Notations are as follows:

	Weight	Density	Concentration of soluble proteins
n'the supernatant	l_n	d_n	C_n
n'th precipitate	p_n	ρ_n	C'_n

Approximately it can be assumed that the supernatant contains no solid and the precipitate contains no liquid phase. If the partition of proteins between the liquid and the solid phases is constant, the partition coefficient, K, will be:

$$K=C_n/C_n' \quad\cdots\cdots\cdots\cdots\quad (1).$$

Using a solvent of m proportion to the tissue brei to be extracted, we can obtain from the relations of weight, volume, and protein content the following equations:

$$P_{n-1}+mp_{n-1}=l_n+p_n \quad\cdots\cdots\cdots\cdots\quad (2),$$

$$p_{n-1}\left(\frac{1}{\rho_{n-1}}+\frac{m}{d_0}\right)=\frac{l_n}{d_n}+\frac{p_n}{\rho_n} \quad\cdots\cdots\cdots\cdots\quad (3),$$

$$C'\frac{p_{n-1}}{\rho_{n-1}}=C\frac{l_n}{d_n}+C'\frac{p_n}{\rho_n} \quad\cdots\cdots\cdots\cdots\quad (4),$$

where, d_0 is the density of the solvent. From these equations the rate, K', of the decrease in the extracted protein concentrations (briefly "extraction rate") can be calculated as follows:

$$K'_{n-1}=C_n/C_{n-1} \quad\cdots\cdots\cdots\cdots\quad (5),$$

$$K'_{n-1}=\frac{(\sigma_n-d_n)d_0}{d_0\rho_{n-1}(1+m)\ (1-K)-(d_0+m\rho_{n-1})\ (d_n-K\rho_n)}. \quad (6).$$

If, as a practice, the solvent added is the physiological saline of the same quantity as the tissue brei, then:

$m=1$, and $d_0=1$.

Further, when the partition coefficient, K, is 1, it becomes:

$$K'_{n-1}=\frac{1}{1+\rho_{n-1}} \quad\cdots\cdots\cdots\cdots\quad (7).$$

The density, ρ_n, of the precipitate decreases as the extraction succeeds. The density, however, is usually of the order of 1.01 to 1.1, so that the effect of the variation of density on K' is almost negligible. Hence, as a first approximation, we can assume that the density of the precipitate is constant:

$\rho_n \fallingdotseq \rho$.

Thus, on account of this assumption, the extraction rate, K', is constant, and will be:

$$K'=1/(1+\rho) \quad\cdots\cdots\cdots\cdots\quad (8).$$

And the concentration of the (n+1)th supernatant, C_{n+1}, can be given by the following equation:

$$C_{n+1}=C_1K'^n \quad\cdots\cdots\cdots\cdots\quad (9).$$

Logarithmically expressed:

$$\log C_{n+1}=n\log K'+\log C_1 \quad\cdots\cdots\cdots\cdots\quad (10),$$

this expression makes clear the linear relation between the number of the successions of extraction and the logarithm of the concentration of the supernatant.

2. *Differential and Integral Extraction*

Where the extraction rate is constant, the quantity of the total extractable proteins can be calculated as follows:

The total quantity extracted in the first step, D_1, is: $D_1=C_1\times l_1$.
That in the second step, when all the precipitates obtained in the first step were used, will be:

$$D_2=C_2\times l_2\times\frac{2p_1}{l_2+p_2}.$$

That in the n'th step:

$$D_n=C_n\times l_n\times\frac{2p_1}{l_2+p_2}\times\frac{2p_2}{l_3+p_3}\times\cdots\cdots\cdots\times\frac{2p_{n-1}}{l_n+p_n}.$$

The total quantity ultimately extractable from the quantity of the tissue used is the sum of the quantities extractable in all the steps up to the infinite. The former may be assigned as the integral and the latter as the differential extraction. As the extraction succeedes, the quantity of the extracted protein decreases rapidly, and the quantity of the supernatant and that of the precipitate will become nearly equal. Hence, where all the precipitates obtained in the preceding step were used, the differential extraction from a definite step onward would decrease with the same rate as the extraction rate, K'. If this step is *i*'th, the integral extraction will be:

$$I=\sum_{n=1}^{i-1}D_n+\frac{D_i}{1-K'}.$$

EXPERIMENTAL

Method and Material

Bovine liver, kidney, or lymph-nodes washed with 0.9 per cent sodium chloride solution and removed of fatty and connective tissues were first chopped with scissors and then homogenized twice for five minutes with a cooled blade homogenizer. To the tissue brei the same quantity of 0.9 per cent saline solution was added and again homogenized for five minutes. The tissue homogenate was centrifuged for 20–30 minutes at 3000 r.p.m. To the precipitate obtained was added the same quantity of the saline solution. Then the mixture was homogenized and centrifuged again. The extraction usually proceeded successively to the fourth or fifth stage.

The total nitrogen content of each supernatant and its filtrate was estimated by the usual semimicro-Kjeldahl method. The filtration of the supernatant was performed in an ice-box overnight with No.4 Tokyō filter paper. The density of the precipitate was measured with a pycnometer.

The inorganic phosphorus was estimated by **Allen**'s method (*4*).

Electrophoreses were performed with a Hitachi medium type Tiselius' apparatus (schlieren diagonal system), using a 9 cm.-cell. Protein solutions for analyses were previously dialysed against phosphate buffer of pH 7.8 and ionic strength of 0.1 or 0.05 for 48 hours. Relative concentrations of the components were computed by weighing the enlarged photograms.

I. Liver and Kidney

Experimental results are summarized in Tables I and II. In Fig. 1, logarithms of the concentrations in total nitrogen of liver and kidney extracts are plotted against the number of the successions of the extraction. Fig. 1. shows clearly that the linear relationship is kept between them in the range of experimental error.

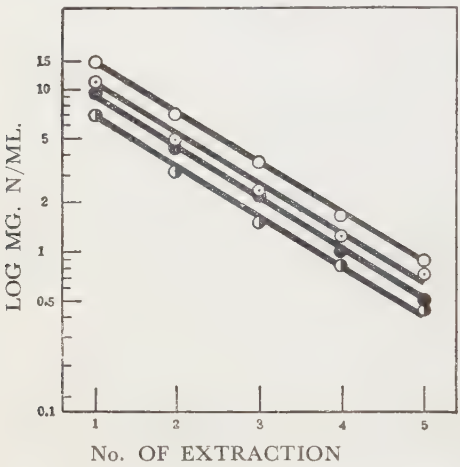

Fig. 1. Successive extraction of nitrogen from liver and kidney.
—○—○— Supernatant (Liver)
—⊙—⊙— Filtrate (Liver)
—●—●— Supernatant (Kidney)
—◑—◑— Filtrate (Kidney)

The density of the precipitate is in the range from 1.03 to 1.05. From the values of density, the extraction rate of the extracted nitrogen, K′, is calculated as 0.43 to 0.48. The coincidence of the values of K′ obtained with those calculated is fairly good.

On account of this coincidence, it can be inferred that the partition coefficient of proteins between the supernatant and precipitate is unit, *i.e.*, the solution remaining in the precipitate is extracted successively thereafter. Therefore, we may also infer that each successive extract will show similar electrophoretic patterns, or the relative concentrations of electrophoretic components in them will be constant. In Figs. 2 and 3, the electrophoretic patterns, and in Tables III and IV, the mobilities and relative concentrations of the main electrophoretic components of the first and the second extracts of liver and kidney are shown, respectively. The values of mobilities are the means of three runs and those of relative concentrations are computed from one individual run, each individual run showing slight deviations. The results show that the relative concentrations of liver protein components remain constant, and those of kidney also nearly constant notwithstanding the number of successions of the extraction.

Total Nitrogen and Protein Contents—We have measured only the total nitrogen content and used it as a measure of the protein content. Usually the protein content is measured by precipitating the proteins by a suitable precipitant. The degree of the precipitation, however, varies as the precipitant varies. Further, the total nitrogen content of liver and kidney is far larger than their non-protein nitrogen content, and the extraction rate of N. P. N. would be the same as that of the proteins, so far as K=1. For these reasons we have used the total nitrogen as am measure of the protein content.

The Partition of the Soluble Tissue Proteins—In the theoretical treatment, the super-

TABLE I

Extraction Rate of Nitrogen of Liver and Kidney

Liver					
No. of extraction	1	2	3	4	5
C_n (Supernatant) *mg.*N/*ml.*	14.9	7.0	3.5	1.7	0.89
K_n'1)	0.47	0.50	0.48	0.53	
C_n (Filtrate) *mg.*N/*ml.*	11.1	4.9	2.4	1.24	0.73
K_n'	0.44	0.48	0.51	0.59	
% (Filtrate)2)	75	70	68	74	81
P_n'3)	0.76	0.72	0.82	0.96	1.0
Kidney					
C_n (Supernatant) *mg.*N/*ml.*	9.5	4.4	2.3	1.00	0.53
K_n'1)	0.47	0.51	0.45	0.53	
C_n (Filtrate) *mg.*N/*ml.*	6.9	3.2	1.6	0.82	0.43
K_n'	0.46	0.50	0.53	0.52	
% (Filtrate)2)	73	72	69	82	80
P_n'3)	0.86	0.84	0.84	0.98	0.96

1) $K_n' = C_{n+1}/C_n$

2) Obtained by the ratio, C_n (Filtrate)/C_n (Supernatant).

3) Ratio of the precipitate obtained to the precipitate used for extraction.

TABLE II

Mobilities and Relative Concentrations of the Main Electrophoretic Components of Liver Extracts

No. of peak	Mobilities1)		Relative concentrations	
	No. of extraction		No. of extraction	
	1	2	1	2
1	9.3	9.4	2.5%	2.5%
2	7.3	7.8	13.7	12.9
3	5.6	6.0	36.8	34.8
4	3.6	3.9	27.3	28.1
5	1.6	1.6	19.7	21.7

Calculated with the ascending limbs.

Phosphate buffer of pH 7.8, ionic strength of 0.05 was used.

Voltage gradient, 7.9 volt/cm.

1) $\times 10^{-5}$ cm^2./volt, sec.

TABLE III

Mobilities and Relative Concentrations of the Main Electrophoretic Components of Kidney Extracts

No. of peak	Mobilities1)		Relative concentrations	
	No. of extraction		No. of extraction	
	1	2	1	2
			%	%
1	8.4			
2	6.9	6.3	2	1
3	5.7	5.6	16	8
4	4.7	4.5	18	53
5	4.0	4.3		
6	3.5	3.7	30	
7	2.7	3.3		
8	2.3	2.5	13	19
9	0.8	1.0	21	19

Calculated with the ascending limbs.
Phosphate buffer of pH 7.8, ionic strength of 0.1 was used.
Voltage gradient, 6.1 volt/cm.
1) $\times 10^{-5}$ cm$_2$./volt, sec.

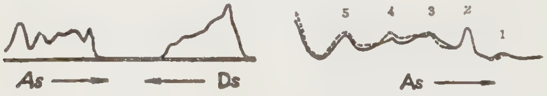

FIG. 2. Electrophoretic patterens of liver extracts
—— First extract.
---- Second extract.
Phosphate buffer at pH 7.8, ionic strength of 0.05 was used.
Voltage gradient, 7.9 volt/cm.

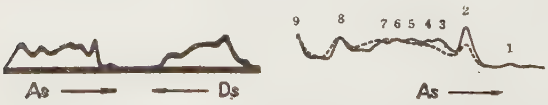

FIG. 3. Electrophoretic patterns of kidney extract
—— First extract.
---- Second extract.
Phosphate buffer of pH 7.8, ionic strength of 0.1 was used.
Voltage gradient, 6.1 volt/cm.

natant was assumed not to contain solid and the precipitate not to contain liquid phase. According to our experimental conditions, however, the supernatants contained the particles, amounting to 30 per cent or more of their total nitrogen content, and the precipitate contained a liquid phase. Nevertheless, the experimental results coincide with the theory, which assumed $K=1$. This indicates clearly, as will be discussed later, that the phenomenon treated theoretically as a partition can be regarded as a simple retention of the solution in the network of cell debris in the precipitate. In such a case, the quantity of the solid phase or the "column bed" influences the extraction rate, K′, only mechanically, as will be fully discussed elsewhere (*5*). Thus:

$$K'\frac{1-\sigma}{1+\rho-\sigma} \cdots\cdots\cdots\cdots (11),$$

where σ is the volume proportion of the insoluble solid phase in the precipitate. The equation as a result shows that if σ is large, K′ will be definitely smaller than 0.5.

On the other hand, the contaminating rate of the solid in the supernatant will hardly influence the extraction rate, K′, provided the quantity of the solid can be assumed to be proportional to the protein concentration of the supernatant. For, if α is the proportion of the solid represented by the nitrogen concentration, the nitrogen concentration, C_{0n}, of the supernatant without the solid will be approximately:

$$C_n=C_{0n}\ (1+\alpha).$$

Therefore, the extraction rate, K′, remains constant:

$$K'=\frac{C_{n+1}}{C_n}=\frac{C_{0(n+1)}}{C_{0n}}\ .$$

In the above discussion, the Eq. (11) was based on the assumption that the contaminating solid were proportional to the protein concentration of the supernatant, and this was practically the case presented here, in so far as the extraction rate was constant. But when the successive extraction proceeds and the protein concentration of the supernatant decreases, this relation fails to hold. For here the protein concentration of the supernatant becomes so small that it will be definitely affected by the slight contamination caused by the solid particles which is rich in nitrogen. The trend of the extraction rate to increase as the number of successions proceeds, will partially be explained by this reason. The filtration to obtain a pure supernatant, which is rather a primitive procedure, tends to entangle the condition further. Hence it seemed not practical to prolong the extraction beyond the fifth extraction.

The partition of a substance between two phases is effected by the diffusion of the substance. Considering the macromolecules of proteins, it is uncertain whether their partition could be completed in the time employed in this present experimental conditions. As can be seen from the results, the successive extraction could be treated with the concept of the partition of proteins between the solvent and the tissue debris. But so far as the partition coefficient, K, is 1, the phenomenon can be explained as well by the mere retention of the solution in the precipitate, not to mention the adsorption. When, however, K, is smaller than 1, or the rate, K′, is larger than 0.5, the retention of the solution in the precipitate must be ruled out.

The electrophoretic studies of the liver and kidney extracts were performed by several workers *(6–11)*. Some differences found between the experimental results of theirs and ours must be further investigated as to the experimental conditions. The chief draw-back in our experiments is the insufficient centrifugation. It obscures the elucidation of the extraction rate of the proteins, and disturbs the electrophoretic studies by necessitating the prolonged filtration. We are now preparing for the experiments with a high speed centrifuge.

II. Lymph-node

The experimental results with the bovine lymph-nodes are summarized in Fig. 4. The results were not always alike, but in every case a discontinuity of the extraction rate, K′, of nitrogen was observed between the first and the second or between the second and the third extraction, where the extraction of nitrogen increased. When the tissue was mildly homogenized, the step of extraction where the discontinuity occurred could be retarded. Nevertheless the extraction rate of inorganic phosphorus remained nearly constant. Consequently the discontinuity observed in the nitrogen extraction seems to be due to some biological reactions, possibly the caryolysis. But it requires further investigations for the final conclusion.

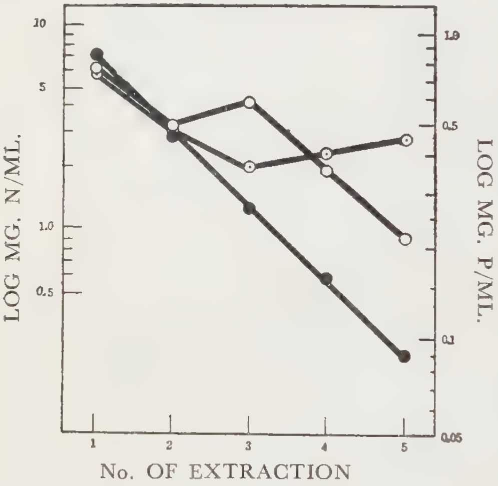

Fig. 4. Successive extraction of nitrogen of lymph-node.

—○— Ordinary extraction.

—⊙— Homogenization retarded by shortening the time of homogenization to 1 minute.

—●— Extraction of inorganic phosphorus.

In this connection a few results are interesting, of which the one is the volume change of precipitates, and the other the electrophoretic patterns.

As to the former, the results presented in Fig. 5 are based on the weight of the precipitates, for the increases in weight is evidently due to the change of volume. Curve 1 in Fig. 5 indicates the real recovery of the precipitate per homogenate used in each step and Curve 2 the calculated quantities obtainable from 100 g. of the lymph-nodes. The actual decreases in the quantity of the successive precipitate owing to the extraction cannot be known, but assuming the second and the third precipitates to decrease with the rate proportional to those of the first and the fourth (represented by the dotted line), the rate of volume change in each step would be represented by Curve 3. From this, it can easily be seen that the volume

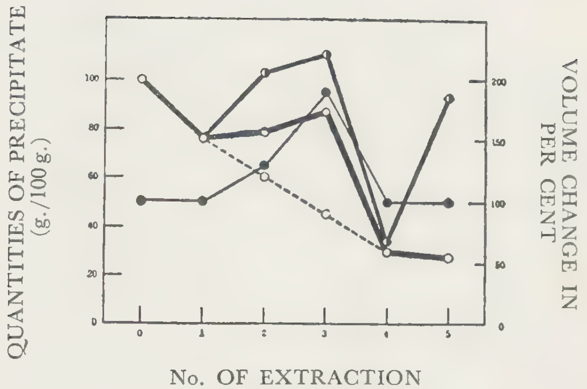

Fig. 5. Volume change of the successive precipitate of lymph-node.

—○— Curve 1. Quantities of the successive precipitates obtained from 100 g. of the original homogenate used.

—◑— Curve 2. Quantities of each successive precipitate obtained from 100 g. of each previous precipitate used.

—●— Curve 3. Volume change of the successive precipitates calculated from Curve 2, taking the values on dotted line as 100%.

of precipitate increases from the second to the third step more and more, though in the fourth step decreased suddenly.

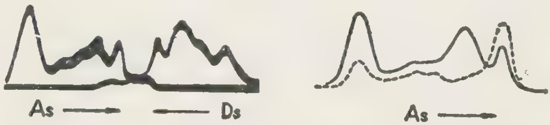

Fig. 6. Electrophoretic patterns of the extract of lymph-node

—— First extract.

- - - - Second extract.

Phosphate buffer of pH 7.8, ionic strength of 0.05 was used.

Voltage gradient, 8.1 volt/cm.

As can be seen from Fig. 6, the electrophoretic patterns of the first and the third extracts clearly differ from each other. The patterns of the extract of lymph nodes were also studied by several other workers (*12–15*). If the patterns reported are different by workers, it would be due to the sort of animal used and the extraction procedure adopted. The results presented here show that some biological reaction can occur and influence the extraction of proteins during the procedure.

SUMMARY

1. When the tissue brei is extracted with the same quantity of physiological saline solution, the rate of the nitrogen concentration of the two successive extracts, briefly the extraction rate, K′, will be:

$$K' = 1/(1+\rho),$$

where, ρ is the density of the precipitates, provided neither partition nor adsorption of proteins takes place.

2. Above relation was confirmed in the cases of liver and kidney.

3. In the case of lymph-node, a discontinuity of the extraction rate was observed, which could be ascribed to some biological change during the extraction procedure.

4. Relative concentrations of the main electrophoretic components of liver and kidney remained constant in the successive extracts. Those of lymph-nodes changed with the change of the extraction rate.

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SUCCESSIVE EXTRACTION OF TISSUE PROTEINS

II. BRAIN AND INTESTINAL MUCOSA

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(Received for publication, September 15, 1953)

As to the extraction of the proteins of the brain, various solvents were employed by Palladin *et al.* (*1*). As has previously been reported (*2*), however, by means of extraction of tissue, the process of the extraction of the tissue proteins can investigated. The experimental results were interpreted in terms of the extraction rate, K′, the rate of decrease in the extractable nitrogen of each following extract. The proteins of liver and kidney were found to be dissolved in the physiological saline thoroughly by the first homogenization and not adsorbed by the tissue debris.

It can be seen from the present investigation that the same can also be said in the case of brain. However, the extraction rate of nitrogen of the brain is definitely smaller than that expected from the density of the precipitate, a fact which would be accounted for by the effect of the volume of insoluble material. In both cases partition coefficient of proteins can be assumed to be unit, *i.e.* no partition to take place.

If the extraction rate is larger than 0.5, or more precisely, larger than that expected from density as calculated from Eq. (*7*) in the preceding paper (*2*), it can no longer be accounted for by the volume effect of insoluble tissue debris. The extraction of the intestinal mucosa itself reveals this case.

EXPERIMENTAL

Method and Material

Brain and Lung—Experimental methods are the same as described previously (*2*). A hemisphere of bovine cerebrum was washed two to three times with 0.9 per cent sodium chloride solution and the meninges with blood vessels were removed as much as possible. A bovine lung was washed and irrigated with about 3 liters of physiological saline solution immediately after slaughter. The lung become somewhat pale, but the blood could not thoroughly be washed out. The brain was first chopped with scisors and the lung first minced, then homogenized 10 minutes with a cooled blade homogenizer. To the tissue brei, the same quantity of physiological saline was added and again homogenized for 5 minutes. The homogenate was weighed and centrifuged for 30 minutes at 3000 r.p.m. The precipitate obtained was again homogenized with the same quantity of physiological saline and centrifuged. And so on.

Intestinal Mucosa—About one meter of oral part of intestine jejunum was washed with tap water thoroughly and after the connective and fatty tissues were removed the wall was cut open and washed twice with 0.9 per cent saline solution. The mucosa was scraped off with the edge of a slide-glass and homogenized with a cooled blade homogenizer. The brei of the mucosa was successively extracted with the same quantity of physiological saline solution, in the same manner as for the other tissues.

Various methods of analyses were the same as described in the previous report (*2*): Total nitrogen was measured by the usual semimicro-Kjeldahl method. The filtration of the supernatant was performed in an ice-box overnight with No. 4 Toyō filter paper. Density of the precipitate was measured with a pycnometer.

Electrophoretic analyses were performed with a Hitachi medium type Tiselius' electrophoretic apparatus (schlieren diagonal system), using a 9 cm.-cell. Protein solutions for analyses were previously dialyzed against phosphate buffer of pH 7.8 and ionic strength of 0.05 for 48 hours.

Estimation of the added xylose was carried out by the method of Hagedorn (*3*), and that of the added iodide by the method of Ogata and Hayashi (*4*). The added *p*-nitrophenol (PNP) was estimated colorimetrically, refering to the method reported by Morimoto (*5*) as follows: To 1 ml. of the supernatant containing PNP was added 9 ml. of 10 per cent trichloracetic acid and filtered. To 2 ml. of the filtrate 4 ml. of saturated sodium carbonate was added, and the color obtained was estimated by an electrophotometer.

I. Successive Extraction of Brain and Lung.

Experimental results are summarized in Table I.

Extraction Rate—The theory of successive extraction by Nakamura *et al.* predicts that the extraction rate, K′, of the n'th supernatant can be given by the equation:

$$K'_n = 1/(1+\rho_n) \qquad (1),$$

where, ρ_n is the density of the n'th precipitate and can be substituted by the mean density of the successive precipitate, provided K′ is constant, and the partition coefficient, K, is a unit, *i.e.* the partition of proteins between the solution and the precipitate does not take place. When this equation is applied to the obtained values of densities of both brain and lung, the extraction rate, K′, is calculated to be 0.495 in both, since the density of the precipitate is about 1.01–1.02 in both cases.

The extraction rate obtained experimentally from the lung coincides fairly well with that calculated. The rate of the brain, however, differs definitely from those calculated.

The Insoluble Material in the Tissue—The theory by Nakamura *et al.* is, as was discussed previously, based on the assumption that the precipitate contained only substances which partake in the partition or adsorption of proteins, or that the bulk

TABLE I

Extraction Rate of Nitrogen of Brain and Lung

Brain	No. of extraction			
	1	2	3	4
C_n, *mg.* N/*ml.*	4.4	1.95	0.62	0.26
$K_n'^{1)}$	0.44	0.32	0.42	
$P_n'^{2)}$	1.32	0.90	1.04	1.04
Lung				
C_n, *mg.* N/*ml.*	5.1	2.5	1.4	0.6
$K_n'^{1)}$	0.49	0.54	0.45	
$P_n'^{2)}$	0.94	0.98	1.0	1.08

1) $K'_n = C_{n+1}/C_n$

2) Ratio of the precipitate obtained to the precipitate used for extraction.

of the insoluble solid in the precipitate is negligible. In reality, however, there exist in the homogenates inert insoluble substances which have no concern in the partition nor solubility for the proteins. If the volumes of these insoluble material are taken into account, the effective volume of the precipitate which partakes in the partition or solution of proteins must be reduced by them. It would be comparable to the state where the precipitate was compressed into the volume without the inert portion. Thus, if the weight and density of the inert material are S and ρ_s, respectively, the density, ρ' which affects K' will apparently be:

$$\rho' = \frac{P_n}{P_n/\rho_n - S/\rho_s} = \frac{P_n \rho_n \rho_s}{P_n \rho_s - S \rho_n} \quad \cdots\cdots \quad (2).$$

As an approximation $\rho_s = \rho_n$, so the Eq. (1) will be:

$$K'_n = \frac{1-\sigma_n}{\rho_n + 1 - \sigma_n} \quad \cdots\cdots \quad (3),$$

where $\sigma_n = S/P_n$. Thus, for the calculation of the extraction rate the quantity of the inert solids, S, or the rate, σ, of the inert solid in the precipitate must be known.

Theoretically, we can calculate, σ, of inert solid in the precipitate, using Eq. (3) and the extraction rate of nitrogen, but as the extraction rates obtained experimentally vary considerably, it would not be reasonable to use them for the calculation. Hence, it seemed preferrable to add a known quantity of a suitable substance to measure its extraction rate. For such substances to be added, it is necessary that, (a) there is no interaction between the substance added and tissue components, (b) to use as small a quantity as possible, that the microestimation is possible, and (c) the estimation procedure is simple. We selected xylose, *p*-nitrophenol (PNP), and potassium iodide for such substance.

Xylose was taken as a reducing sugar, and the results were obscured by the other reducing substances contained in the tissue.

p-Nitrophenol interacted with the component of brain. As will be discussed elsewhere (*6*), it is probably adsorbed on the insoluble residue of brain homogenate. Only potassium iodide served the purpose. For, whereas iodine combines with proteins and lipides, iodide, as is known, does not. As the results presented in Table II show, the extraction rate of iodide is nearly constant, indicating the indifference of the iodide. The value of σ is about 0.2, calculated from K′ of the iodide added.

TABLE II

Extraction Rate of Added Potassium Iodide

No. of extraction	0	1	2	3
K_n' 1)	0.57	0.45	0.43	0.45
P_n' 2)		1.4	0.9	1.0
σ	0.25	0.18	0.25	0.18
K_n, calcd. 3)	0.56	0.46	0.46	0.46

1), 2) As in Table I.

3) The values of K_n' were calculated by assuming $\sigma=0.2$.

The direct method of calculating σ is to compute the volume of the residue remaining ultimately after successive extractions, from its density and its nitrogen content. The density, however, cannot be measured directly. Assuming no volume change to take place by drying, or that the density approximates unit, the value of 0.2 can be obtained from the nitrogen content of 55 mg. per g. of the final residue and the content of 1.1 g. of insoluble substances per 100 g. original brei.

From the values of σ computed from the extraction rate of iodide and from the nitrogen content of the solid, the value of K′ can be calculated as 0.46, which differs from that of extractable nitrogen presented in Table I. This shows that the decrease of the extraction rate of nitrogen cannot sufficiently be accounted for by the rate of solid in the tissue brei. Most probably it would depend upon the contamination of the supernatant by the solid. If this is the case, following successive extractions will merely result in the extraction of the solution retained in the preceeding precipitate, and each successive extract will present the constant relative concentrations of their components. In Fig. 1 the electrophoretic patterns and in Table III the mobilities and relative concentrations of the main electrophoretic components of the first and the second successive extracts are shown. It can be seen from the results cited that the relative concentrations of the components remain practically constant. Hence, it follows that the successive extraction of brain homogenate is merely the extraction of the preceding extract retained in the precipitate, and no real partition nor adsorption of proteins between them takes place. In any case, it would be necessary to repeat the experiments with a high speed centrifuge.

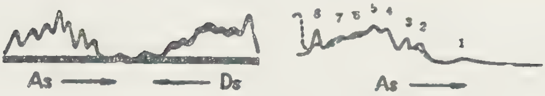

FIG. 1. Electrophoretic patterns of Brain extract.
——— First extract.
------ Second extract.
Phosphate buffer of pH 7.8, ionic strength of 0.05 was used.
Voltage gradient, 8.0 and 7.5 volt/cm. with first and second extracts, respectively.

TABLE III

Mobilities and Relative Concentrations of Electrophoretic Components of Brain Extracts

No. of peaks	Mobilities1)		Relative concentrations	
	No. of extraction		No. of extraction	
	1	2	1	2
1	12.5	11.5	3.2%	3.2%
2	9.6	10.0	7.6	6.0
3	8.2	8.7	8.8	5.7
4	6.8	8.0	19.5	17.9
5	5.6	6.7	19.0	31.9
6	4.0	4,9	18.6	18.1
7	2.6	1.9	15.7	10.9
8	0.1	0.4	7.6	6.3
Voltage gradient, volt/cm.	8.0	7.5		

Calculated from the ascending limbs.
Phosphate buffer of pH 7.8, ionic strength of 0.05 was used.
1) $\times 10^{-5}$ cm^2./volt, sec.

The insoluble nitrogen of the brain amounts to about 1.1 g. per 100 g. and that of the lung also about 1.1 g. Thus the inextractable nitrogen in the lung is the same as or a little larger than that in the brain. Notwithstanding this, the extraction rates of brain and lung differ definitely: that of lung coincides with that expected from the density of the precipitate. This can be accounted for by the difference in the nitrogen content of the insoluble tissue debris of brain and lung. The insoluble material of the brain contains a considerable amount of lipides so that the nitrogen content of the insoluble material is relatively small (5 mg. per g.). On the other hand, as the insoluble substance of the lung consists largely of proteins of about 16 per cent nitrogen content, the content of insoluble protein of the lung would be about 8 per cent, and its volume would amount to 6 per cent, provided the specific volume of the protein is assumed to be 0.73. In respect to our experimental conditions errors of 5 per cent or thereabouts are quite permissible. Hence it follows that the insoluble material in the lung has apparently no effect on the extraction rate, which agrees, well with the experimental results.

II. Successive Extraction of Intestinal Mucosa

Experimental results are summarized in Table IV. Extraction rates in each step are practically constant, so that the linear relationship holds between the logarithms of the nitrogen contents and the numbers of succession.

TABLE IV

Extraction Rate of Intestinal Mucosa

No. of extraction	1	2	3	4	5
Cn (Supernatant) *mg.*N/*ml.*	7.0	4.1	2.6	1.6	0.95
Kn′	0.59	0.64	0.62	0.59	
Cn (Filtrate) *mg.*N/*ml.*	5.5	3.2	1.9	1.2	0.72
Kn′	0.58	0.59	0.63	0.60	
% (Filtrate)1)	79	78	73	76	
Homogenization prolonged to 15 minutes.					
Cn (Supernatant) *mg.*N/*ml.*	7.1	4.1	2.5	1.6	1.0
Kn′	0.58	0.61	0.64	0.62	

1) Obtained by the ratio, Cn(Filtrate)/Cn(Supernatant).

As the densities of the precipitates lie in the range of 1.01 to 1.02, the value of the extractson rate expected from the Eq.(1) must be 0.49 to 0.50. On the other hand the value of K′ obtained experimentally averages 0.62. This discordance cannot be accounted for by the volume of the insoluble substance, as in the case of the brain, for otherwise the volume should be negative in this case. Therefore, the discordance must rely either on the experimental conditions or on the extractability of the proteins *per se*.

Of the experimental conditions employed here, the most uncontrollable ones are the homogenization and the centrifugation. The amount of the particules remaining in the supernatant depends on the degree of centrifugation and must influence considerable the extraction rate, as the nitrogen content of the particulate is far higher than that of the extract. The extraction rate, however, of the filtrates obtained from the supernatants is nearly equal to that of the latter, as shown in Table IV. Hence the amount of the particulate remaining in the supernatant is nearly constant, *i. e.* the quantity of the particulate in it appears to be proportional to its concentration in the protein. At any rate, in this respect also, it is necessary to repeat the experiments with a high speed centrifuge.

The extent of cell destruction depends on the degree of homogenization. If the destruction in the first homogenization were incomplete and barely completed in several steps, the second supernatant would contain more proteins than would be expected from Eq. (1), *i. e.* from the standpoint of the mere dilution of previous solution retained in the precipitate. When, however, the duration of the homogenization was varied experimentally, the extraction rate, K′, still remained unaltered, as can be seen in Table IV. Hence the destruction of cells is practically complete under the present experimental conditions.

As long as the experimental condition which would cause the extraction rate increase could be ruled out, the extractability of the proteins, *per se*, remains as the only possible cause. It is, however, obscure whether it depends on the partition or on the adsorption of the proteins. In any case, the excessive extraction rate means that the successive extraction of the intestinal mucosa is not the mere dilution of the previous extract retained in the precipitate, as was the case with liver, kidney, and brain, but the dissolution of some additional proteins. If this is actually the case, it will be revealed in the electrophoretic patterns of the successive extracts. For it is improbable that the additionally dissolved proteins would distribute evenly among the electrophoretic components and so their relative concentration would remain unaltered.

Electrophoretic Components of Intestinal Mucosa—Electrophoretic patterns are presented in Fig. 2, mobilities and relative concentrations of the electrophoretic components in Table V. In the patterns of the ascending and the descending limbs, the number and the shape of peaks are obviously different. As discussed by Longsworth *et al.* (7), the interactions of components take place in such a case and the components and their mobilities in either limb differ from each other. Therefore, two components with the same mobility on either side are not always identical. The identical component, on the same limb, however, will show the same mobility, even if its relative concentration is different in each run. Hence the relative concentrations of the components of successive extracts were compared on the same limb.

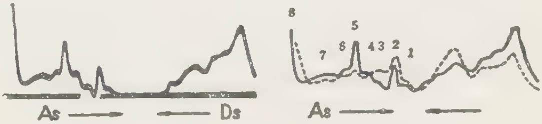

Fig. 2. Electrophoretic patterns of the extract of Intestinal Mucosa

– —— First extract.

------ Second extract.

Phosphate buffer of pH 7.8, ionic strength of 0.05 was used.

Voltage gradient, 9.0 and 8.6 with first and second extracts, respectively.

TABLE V

Mobilities and Relative Concentrations of Electrophoretic Components of Intestinal Mucosa

No. of peaks	Mobilities[1] No. of extraction 1	Mobilities[1] No. of extraction 2	Relative concentrations No. of extraction 1	Relative concentrations No. of extraction 2
1	13.4	14.4	2%	6%
2	12.0	12.3	15	28
3	9.9	9.8	5	21
4	8.6		8	12
5	7.0	6.7	31	9
6	6.0		8	11
7	3.7	4.5	32	13
Voltage gradient, volt/cm.	9.0	8.6		

Phosphate buffer of pH 7.8, ionic strength of 0.05 was used.

Calculated with the ascending limbs.

1) $\times 10^{-5}$ cm.^{2}/volt, sec.

As can be seen from Table V, relative concentrations of the electrophoretic components of the intestinal mucosa vary considerably as the extraction proceeds from the first to the second step, and this can also be visualized by the patterns shown in Fig. 2.

In this connection, if the extraction rate of a tissue could be altered in some way, the method would serve, as it were, as " extraction chromatography."

SUMMARY

1. The extraction rate, K′, *i.e.* the rate of the decrease of the extractable nitrogen in the successive supernatants, is, smaller in the case of brain, and larger in the case of intestinal mucosa, than that expected from the following equation:

$$K' = 1/(1+\rho).$$

2. The smallness of the extraction rate of brain can be ascribed to the effect of insoluble substance, and the excess, in the case of intestinal mucosa, to the extractability of the proteins.

3. Results of electrophoretic studies of the physiological saline extract of brain and intestinal mucosa are presented.

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The Journal of Biochemistry, Vol. 41, No. 1, 1954

AFFINITY BETWEEN PHOSPHODIESTERASE AND PHOSPHATE COMPOUNDS

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In the previous paper (*1*) some micromethods for enzymatic studies were reported. Their further application has been undertaken. Phosphodiesterase liberates only one mole of hydroxyl compound from diester of phosphoric acid. The enzyme solution optimally active at pH 7 can be obtained free from other phosphatases (*2*). Among the substrates for this enzyme di-*p*-nitrophenyl-phosphate (DNPP) is one of the most puickly hydrolysable diesters. Diphenyl-phosphate (DPP) can be split far more slowly than the *p*-nitrophenol derivative yet at a moderate rate, while there are some diesters, distinct hydrolysis of which is observable only after a long incubation time, and Michaelis constants of the enzyme complex with such diesters are in general difficult to determine. Those of DPP or DNPP are, however, easy, since the initial rates of their hydrolysis can be estimated precisely by colorimetry of liberated phenol or *p*-nitrophenol. Then pKm in respect to other diesters or even to any phosphate compound could be found, if those substances would competitively inhibit the diesterase action. This paper deals with the results of such experiments.

METHODS

Potassium diphenyl-phosphate and sodium di-*p*-nitrophenyl-phosphate were used as a substrate for diesterase. The latter was prepared through the partial hydrolysis of tris-*p*-nitrophenyl-phosphate, since the solubility of the calcium salt (*2*) was too low for the present experiment. Diesterase solution prepared according to Yoshida (*2*) was already poor in protein, but in order to exclude its slight opalescence sodium chloride at 0.1 per cent and 0.1 M acetic acid were added to the solution to adjust pH to 5 and centrifugated. The supernatant was so clear and colorless that deproteinizing procedure could be omitted before photometry of the reaction products. Composition of the reaction solutions was in general as follows: Two ml. of the substrate solution, 3 ml. of veronal acetate buffer (pH 7), and 2 ml. of water were mixed and preincubated at 37° for some time and to it was added 3 ml. of similarly warmed diesterase solution.

In the experiments for inhibition 2 ml. of any inhibitor solution was used instead of water. For phenol estimation 1 ml. of the reaction mixture was added to 1 ml. of 3 per cent borax solution and after indophenol formation (*1*) the blue solution was diluted to a 10 ml.-mark. To estimate *p*-nitrophenol the solution was added to the same volume of concentrated soda. Photometry was carried out in a 10 mm.-cubet with filter S_{61} and S_{43}. Phenol and *p*-nitrophenol do not disturb the photometry of each other.

RESULTS

Direct Measurement of pKm of the Diesterase Diphenyl-phosphate Complex. (see Table I)—Extinction coefficient was measured at the start and after 1, 2, and 3 hours. The E increase was exactly proportional to the time with an irregular deviation of not over 2 per cent.

TABLE I

Hydrolysis of Diphenyl-phosphate

Final concn. of substrate	Average E increase*	Average hydrolysis	Relative activity**
M/5	0.171	0.038%	1.0
M/10	0.168	0.07	0.98
M/20	0.157	0.13	0.92
M/40	0.142	0.24	0.83
M/100	0.125	0.53	0.73
M/200	0.098	0.81	0.57
M/400	0.071	1.29	0.42
M/800	0.043	1.47	0.25

* Per one hour.

** Calculated from V_∞ (E increase for one hour)=0.171, obtained according to Lineweaver-Burk (*3*).

Activity at various concentrations of the substrate agrees fairly well with the values on a pS curve drawn by assuming pK*m* as 2.45.

Direct Measurement of pKm of the Diesterase Dinitrophenyl-phosphate Complex. (see Table II)—Increase of E was measured after 10, 20 and 30 minutes. Irregular deviation of about 3 per cent at most from each average E increase for 10 minutes was shown.

Indirect Measurement of pKm of Diesterase Diphenyl-phosphate Complex. (see Table III)

TABLE II

Hydrolysis of Di-p-nitrophenyl-phosphate

Final concn. of substrate	Average E increase*	Average hydrolysis	Relative activity**
$M/200$	0.120	0.29%	0.88
$M/400$	0.113	0.54	0.83
$M/800$	0.098	0.98	0.72
$M/1000$	0.093	1.12	0.68
$M/1600$	0.076	1.35	0.56
$M/2000$	0.068	1.67	0.50

* For 10 minutes. ** Calculated similarly as in Table I from $V_x = 0.137$. All values lie nearly on a pS curve of pK*m* 3.30.

TABLE III

Inhibition of DMPP Hydrolysis by DPP

Final concn.	Average E increase*	v_0/v	pKi
$M/2000$ DNPP**	0.068	1.45	2.40
〃 $+M/280$ DPP**	0.047		

* For 10 minutes.

** Concentrations corresponding to K*m* of respective diester. Therefore, in this condition pKi calculated from $v_0/v=1.45$ according to Michaelis-Menten (*4*) should be 2.45, which agrees with the experimental value of 2.40.

Indirect Measurement of pKm of Ciesterase Bound with Various Phosphate Compounds. (see Table IV)—Competitive inhibition of DPP hydrolysis by inorganic phosphate, monophenyl-phosphate, and *p*-nitrophenyl-phosphate was ascertained at first. At the final $M/1000$ concentration of those inhibitors the pS curve of diesterase was in all case merely dislocated toward y axis. K*i* in respect of each inhibitor may be obtained either from v_0/v as mentioned above or from the curves according to formula $S'=Km\ (I+Ki)/Ki$, where I is the concentration of the inhibition and S′ the concentration of the substrate, which corresponds to the midpoint of each dislocated curve. pK*i* values were 2.05, 1.95 and 2.35, respectively. Since inhibition by phosphate compounds was found to be of a competitive nature, K*i* might be in general calculated simply from v_0/v observed, for example at the K*m* concentration of the substrate and a certain concentration of an inhibiting phosphate compound. The experiment was carried out using as the substrate either di-*p*-nitrophenyl-phosphate or diphenyl-phosphate.

TABLE IV

pKi Indirectly Measured

Inhibitor	Substrate	
	DNPP	DPP
Inorganic phosphate	3.32	3.34
Phenyl phosphate	3.49	3.39
p-Nitrophenyl phosphate	2.34	2.30
β-Glycerophosphate	2.90	3.00
Methyl phosphate	1.80	1.80
Diethyl phosphate	2.90	2.90
Pyrophosphate	1.65	1.59
Diphenyl pyrophosphate	3.27	3.27
p-Amino phenyl phosphate	3.25	3.28
o-Amino phenyl phosphate	3.20	3.05

DISCUSSION

The micromethods reported previously (*1*) for the estimation of phenol, *p*-nitrophenol, and inorganic phosphate are very suitable to phosphatase studies. Direct and indirect measurement of K*m* of the phosphatase-substrate complex may be carried out without any difficulty. Indirect measurement is possible only when K*m* of any substrate enzyme complex is already known and the respective substrate is far more easily hydrolysable than the other, K*m* of which is to be measured. Therefore, even if K*m* of the diphenylphosphate diesterase complex is given, the indirect method can not be applied to find K*m* of di-*p*-nitrophenyl-phosphate enzyme complex. From the results mentioned above, one may know that the phosphodiesterase can also form a complex with inorganic phosphate or any phosphate compound and, furthermore, that the affinity of phosphodiesterase with diester is not always greater than with other phosphate compounds, which are essentially never attacked at all by that enzyme. In addition, it was remarkable that diethyl methyl phosphate, which was water-soluble, but resistant to phosphatase, similar to trimethyl phosphate (*5*), did not possess any affinity toward phosphodiesterase since it exerted no inhibition upon hydrolysis of $M/100$ diphenyl phosphate even at its own final concentration of $M/50$. Therefore, it may be concluded that at least one dissociable acid radical of phosphoric acid must remain free in order to combine with the diesterase. A similar result has been obtained in an experiment with phosphomonoesterase.*

What may be said from those results is that phosphatases in general combine with phosphoric acid residue of the substrate. On the other hand, it should be noticed that since the specificity of each phosphatase is strictly absolute, the enzyme should have a second affinity group for combining with a definite phosphate linkage, may it be R-O-P of monoester and diester or P-O-P of pyrophosphate and its diester, according to the specificity of respective enzyme.

The dual affinity of an enzyme is more differentiated in the case of β-hexosidase. As reported previously (*6*, *7*, *8*, *9*) either β-glucosidase or β-galactosidase, so far as it belongs to emulsin-type, combines primarily with glycosidic linkage of the substrates and then secondarily with glucose or galactose residue, respectively, while each enzyme of Taka-type combines at first with glucose or galactose residue and then with the common β-glycosidic linkage. Exsistence in nature of such two types of β-xylosidase has also been ascertained (*10*).

At any rate the successive combination of enzyme with its substrate at two positions should be a general principle of enzyme action ; namely, the formation of primary substrate enzyme complex and then of a secondary complex through mutual combination of the second specific groups of the enzyme and the substrate within that intermediate complex itself. Molecule of bound substrate in the secondary complex becomes thereby so distorted that it may be said that the substrate is in an activated state.

SUMMARY

Diphenyl-phosphate (DPP) competitively inhibited the hydrolysis of di-*p*-nitrophenyl-phosphate (DNPP) by diesterase. Since Km of the enzyme-DNPP complex was easily measurable, Km of enzyme-DPP could be obtained by calculation from v_0/v observed. The value agreed well with the Km measured directly by estimating the initial rate of phenol liberation. Inhibiting action upon diesterase of various

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* Communicated by H. Ichikawa at the 22nd General Meeting of the Japanese Biochemical Society, 1950.

phosphate compounds and inorganic phosphate was also competitive. When affinity toward diesterase was measured indirectly in a similary way using DNPP and DPP as a substrate, K*m* calculated in both cases was the same for each inhibitor. Diethyl methyl phosphate did not show any inhibition. Mechanism of the enzyme action was discussed.

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"ALLITHIAMINE" A NEWLY FOUND DERIVATIVE OF VITAMIN B_1

I. DISCOVERY OF ALLITHIAMINE

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In 1950 we have found a hitherto unknown thermostable anti-thiamine factor in the fern besides a known anti-thiamine factor, thiaminase, which has been reported to be present in fish and shell-fish (*1*, *2*). So a study has been made on the distribution of the factors with similar activity in the plant and animal kingdom.

As the result of this study it was elucidated that a series of thiamine inactivating principles, other than thiaminase, which similarily act on thiamine and render it negative for thiochrome reaction as thiaminase, were widely distributed in the plant and animal kingdom.

What is more interesting, this study clarified the fact that the active principle extracted from allium sativum and other allium species was most outstanding in its activity whereby thiamine has been converted to a compound which was negative to thiochrome reaction *in vitro* but maintained the thiamine effect *in vivo*. Since we felt it to be necessary to separate the so-called anti-thiamine principles into two types with different concepts, we have proposed that the so-called anti-thiamine effect which brings thiamine to a total loss of its effect *in vivo* shall be designated as " inactivation ", as has been popularly called till today, while the anti-thiamine effect which appears only *in vitro* shall be designated as " masking action ".

Further studies have led to a success in isolating the unknown compound produced in the reaction mixture of the extract of *Allium sativum* and thiamine to which we gave the name of " Allithiamine " (*3*). The structure of allithiamine has been determined by T. Matsukawa and S. Yurugi (*4*), our cooperators, who succeeded further in synthesizing it.

The present paper is intended to present the results of our studies on the allithiamine as reviewed briefly and somewhat chronologically.

Biochemical Conditions for the Formation of Allithiamine

1. Necessary Conditions for Occurrence of the Masking Effect of Garlic upon Thiamine: The distilled water extract of ground fresh garlic bulbs, centrifuged at 3000 r.p.m for 20 minutes, gave a turbid fluid. A portion of its supernatant part, 1 ml., was added to 1 ml. of thiamine solution and then mixed with 5 ml. of an adequate buffer solution. The mixture was then incubated at 60° for 30 minutes and, after cooled down, it was mixed with 3 ml. of BrCN and 2 ml. of 30% NaOH (*5*). The thiochrome formed in this mixture was determined fluorophotometrically under the customary manner. A parallel experiment was performed by omitting the extract of garlic. The reaction mixture containing garlic extract was shown to be poorer in its thiamine content than the reaction mixture missing it. This loss of thiamine indicates the amouut of thiamine that has been "masked." It was found that 0.1 g. of garlic was capable of masking as much as about 800γ of thiamine under the optimal condition.

Optimum pH—To decide the optimum pH for the masking action of the garlic, 1 ml. of garlic extract, prepared with 50 parts of water added to one part of garlic, was mixed with 50 γ of thiamine at different pH. The results are shown in Fig. 1.

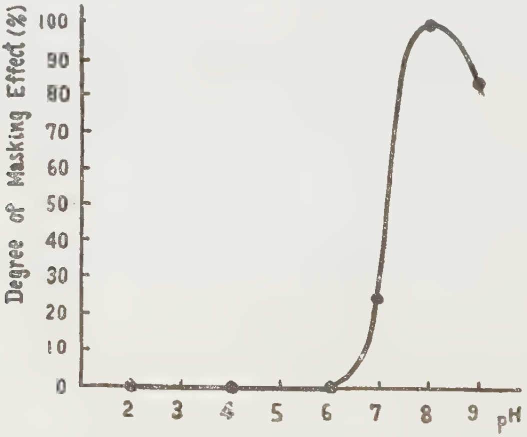


FIG. 1. Influence of pH upon the masking effect of the garlic. Degree of masking effect upon thiamine was represented in percentage proportion to 45 γ, the amount masked at pH 8, assumed as 100 per cent. Amount of thiamine used in this experiment was 50 γ. pH 2, HCl-KCl buffer; pH 4 and 6, acetate buffer; pH 7 and 8, phosphate buffer; pH 9, ammoniac buffer. The reaction mixture (1 ml. of garlic extract, 1 ml. of thiamine solution and 5 ml. of buffer solution) was heated at 60° for 30 minutes.

The masking action was found to occur in alkaline medium above pH 7, the optimum pH being 8.

Optimum Temperature—The similar test has been run at different levels of temperature, namely, room temperature, 38°, 60°, 70° and 80° each for 30 minutes at pH 8. The results are shown in Fig. 2.

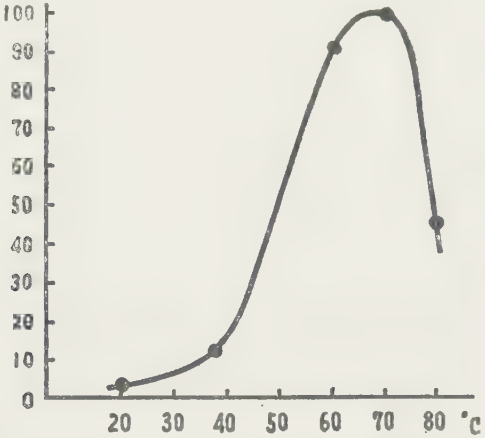


Fig. 2. Effect of temperature on masking action.

Degree of masking effect upon thiamine was represented in percentage proportion to 44 γ, the amount masked at 70°, assumed as 100 per cent. Amount of thiamine used in this experiment was 50 γ.

It was depicted that the masking effect appeared at the level of 60° to 70°, while it could proceed, although very weakly, at the level of human body temperature.

Effect of Oxygen on Masking Action—In order to study the effect of oxygen on the masking action, we have let the reaction proceed in the medium of hydrogen gas and nitrogen gas atmosphere. No retardation occurred in masking effect in either case as shown in Table I. It was established that the masking action did not require the presence of oxygen.

II. Necessary Conditions for Extracting the Active Principle of Masking Action:

While there is no doubt that the extract of ground garlic is capable of masking action, it remained obscure whether the active principle was the intrinsic constituent of tissue of the garlic or a product formed secondarily in the grinding process. In order to give a light to this problem a specimen of extract was prepared from bulbs of garlic boiled for 30 minutes prior to grinding, and was examined for masking effect. As shown in Table II, it was demonstrated that such an extract missed masking effect. However, when such an extract was mixed with a specimen of protein obtained from

TABLE I

The Effect of Nitrogen and Hydrogen Cases upon the Masking Effect of Garlic

Medium		Masked thiamine
Nitrogen gas	+	γ 42
	−	43
Hydrogen gas	+	44
	−	43

Amount of thiamine used in this experiment was 50 γ. Reaction mixture (1 ml. of garlic extract, 1 ml. of thiamine solution and 5 ml. of pH 8 buffer solution) was heated at 60° for 30 minutes in each atmosphere.

TABLE II

Influence of Boiling the Garlic upon the Masking Effect

Method of preparation of the extract	Amount of masked thiamine
Extract of ground fresh garlic	γ 46
Extract of garlic boiled for 30 minutes prior to grinding.	0
Mixture of above extract treated with heat and protein of garlic prepared from fresh material.	41

Amount of thiamine used in this experiment was 50 γ.

garlic, the masking effect was found to revive in the resultant mixture. (The specimen of protein was obtained from the precipitate formed in the extract of fresh garlic when it was acidified till pH 4. The precipitate was washed three times by distilled water. This specimen of protein lacked the masking effect.) This fact suggests that the active principle of the masking action is not an intrinsic constituent of garlic, but a product formed when constituents of garlic tissue are broken down by enzyme activity during the grinding process.

Isolation of Allithiamine

Our study on the necessary conditions for the formation of allithiamine culminated in the success in isolating allithiamine. This success was prompted by the advantageous utilization of the phenomenon that allithiamine shifted almost completely to ether phase when the reaction mixture was shaked with ether. Necessary procedure for the isolation was as follows:

Two-hundred g. of fresh bulbs of garlic (*Allium sativum*) were ground thoroughly, and mixed with 400 ml. of 99 per cent ethanol. The mixture, allowed in a refrigerator overnight and filtered with suction, gave a clear alcohol extract. The residue, extracted further with 400ml. of ethanol, and filtered with suction, gave a filtrate which was added to the previous filtrate to bring the total volume to about 800ml. After this combined filtrate was concentrated to about 50ml. at a low temperature under reduced pressure, 400ml. of 99 per cent ethanol was added to it, and the resultant precipitate was eliminated by centrifugation. The yellow green alcohol extract thus gained was mixed with 1.5 g. of thiamine (dissolved in a small amount of water previously), and, after adjusted to pH 8 with sodium hydroxide (mixture becomes cloudy), it was put in the water bath at 60° for about 1 hour, and pH was adjusted to 8 every 3 to 5 minutes during the cours of time intervening between the 5th to 30th minutes of the incubation, because the mixture was liable to become acid during the progress of the reaction.

After concentrating this mixture at a low temperature under reduced pressure to reduce its volume to approximately 40 ml., and mixing it with 200 ml. of ether, the whole fluid was transferred to a separatory funnel and shaked vigorously so that allithiamine may shift to the phase of ether. The phase of water, looking dark brown, was extracted 3 times more with 100 ml. of ether at each time. After drying the ether fraction, about 500 ml. in total, over anhydrous sodium sulfate ether was distilled off at a low temperature under reduced pressure. When benzene was added to the resulting crude allithiamine and the mixture stirred, feebly yellow rhombic crystall of allithiamine resulted. Recrystallization has been run with benzene.

Structure and Synthesis of Allithiamine

The structure of allithiamine, as determined by our cooperators, T. Matsukawa and S. Yurugi (*4*), is as follows;

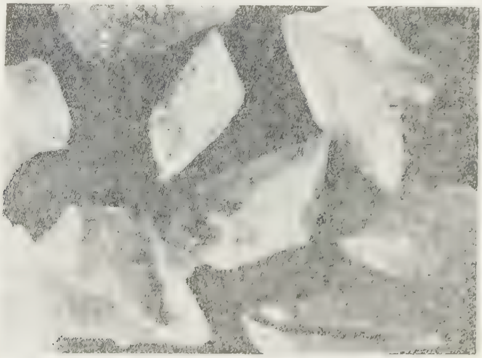
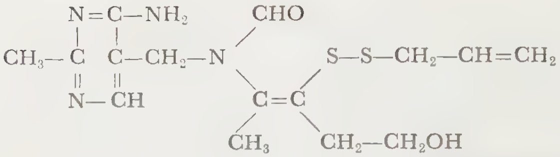


Fig. 3. Allithiamine crystallized from benzene. Magnification 25 diameters.



($C_{15}H_{22}O_2N_4S_2$)
m.p. 132~133° (decomp.)

2-(2′-methyl-4′-amino-pyrimidyl-5′)-methyl-formamino-5-hydroxy-Δ^2-pentenyl-(3) allyl disulfide.

They have proved that the synthesis of allithiamine is possible when we let allicin react with thiamine.

Physicochemical Properties of Allithiamine and Method of Its Quantitative Determination

Physicochemical Properties—Physicochemical properties of allithiamine are as follows: 1) It is easily soluble in absolute ethanol and methanol but slightly soluble in water. 2) Water solution of allithiamine smells like garlic, and tastes bitter. 3) It stimulates thiamine-disulfide (*6*) in major points of its properties. It is negative to thiochrome and diazotized *p*-aminoacetophenon reactions. It is reduced by cysteine, sodium hydrosulphite, sodium thiosulfate and by pieces of liver and other animal organs to thiamine. It is not decomposed by thiaminase and thermostable anti-thiamine factors which are widely distributed in plant kingdom. It is hardly adsorbed by permutit, but adsorbed by fuller's earth in acid.

Quantitative Determination of Allithiamine—Since allithiamine, which is negative by itself to thiochrome reaction, can be made positive to it by the treatment with cysteine or other reducing agents, the following procedure serves as the method of quantitative determination.

1) Determination of allithiamine in the form of a pure solution: One ml. of allithiamine solution, diluted to the concentration of about 0.5 to 1 γ per ml., mixed with 3 ml. of acetate buffer (pH 6) and 1 ml. of 500 mg. per cent cysteine-HCl solution, is heated at 60° for minutes. After cooled down, it is mixed with 3 ml. of BrCN and 2 ml. of 30 per cent NaOH and the resultant thiochrome is measured fluorophotometrically under customary manner. As to reducing effect, application of cysteine-HCl at 60° for 30 minutes was equivalent with application of 10 mg. of sodium thiosulfate at 60° for 20 minutes.

It should be noted that the reduction of allithiamine is influenced greatly by pH level of the reaction mixture. Fig. 4 illustrates the degree of reduction attainable at different levels of pH in case we let 1 mg. cysteine-HCl react with 1.05 γ allithiamine at 60° for 30 minutes.

It has been shown that reduction of allithiamine was best facilitated at pH 7. It follows that increased amount of cysteine-HCl should be used in case pH is apt to get

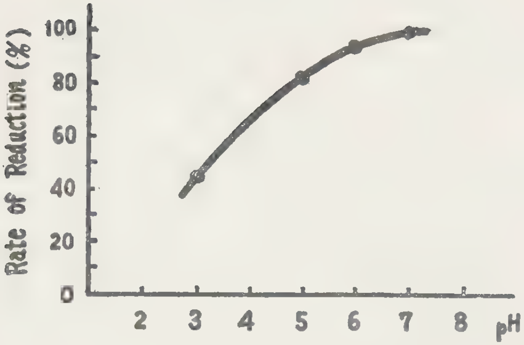


FIG. 4. Influence of pH upon the reduction of allithiamine.

The reduction of allithiamine to thiamine is represented in percentage, 1 γ of thiamine produced being assumed as 100 per cent. Amount of allithiamine used in this experiment was 1.05 γ (equivalent 1 γ of thiamine). Amount of cysteine-HCl added to allithiamine solution was 1 mg. The reaction mixture was incubated at 60° for 30 minutes.

acid.

2) Determination of allithiamine coexisting with thiamine: When an assay is run for thiamine content first, and then subsequent assay is run for total amount of thiamine after reducing allithiamine by some reducing agent, the difference in two assays gives the amount of allithiamine.

3) Determination of thiamine coexisting with thiamine-disulfide: No method is yet available for the differential determination of allithiamine and thiamine-disulfide. But it is possible to distinguish between them qualitatively by means of a paper partition chromatogram. Namely, Rf value is 0.79–0.82 for allithiamine and 0.29 for thiamine-disulfide (acetic acid-butanol-water, 1 : 4 : 5).

4) Determination of allithiamine in the plant or animal tissues: Necessary procedure for determination of allithiamine in the plant or animal tissue simulates the procedure employed for determination of allithiamine coexisting with thiamine. It has been found necessary in most instances to employ permutit for adsorption. It is difficult in general, however, to make a precise determination of allithiamine, since most of the plant and animal tissues tend more or less to reduce allithiamine. Animal tissues, particularly liver or blood above all, exert a marked reducing activity. Fig. 5 illustrates the development of reduction of allithiamine in the mixture of 5 ml. of allithiamine solution and 0.4 ml. of human blood as observed at 20° at different points of time. This figure points the fact that reduction takes place very rapidly.

In order to attain the highest possible precisity in an allithiamine assay, it is recommendable to carry out determination at the lowest possible temperature, adjusting pH level at 1 to 3, and run the assay for thiamine as early as possible prior to reducing

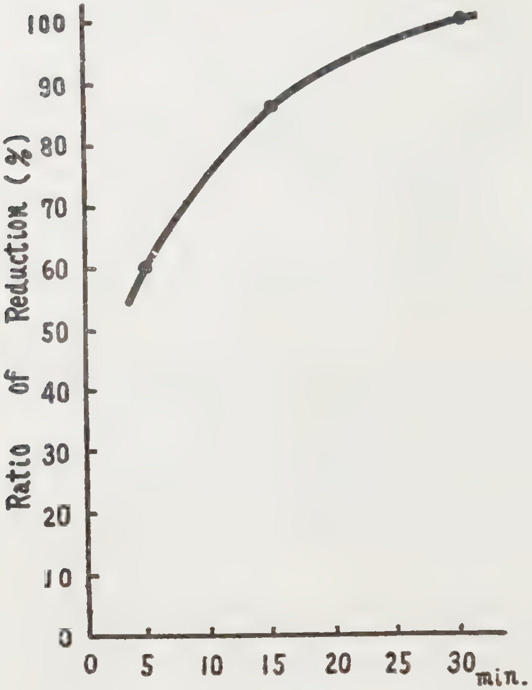


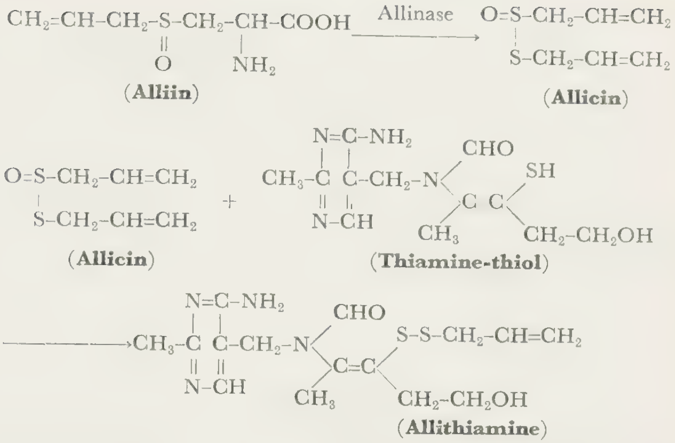
FIG. 5. Reduction of allithiamine by human blood.

Degree of amount of thiamine reduced from allithiamine was presented in percentage, proportion to 1 γ of thiamine, assumed as 100 per cent. Amount of allithiamine used in this experiment was 1.05 γ. Amount of human blood added to allithiamine solution was 0.4 ml.

allithiamine, employing permutite to adsorb thiamine fraction in a given material.

DISCUSSION

Considering the fact that allithiamine has been formed from thiamine and extract of ground fresh garlic but not formed by the extract which is prepared from bulbs of garlic boiled prior to grinding, and that allithiamine has been synthesized from thiamine and pure allicin, there seems to be no doubt that the active principle responsible for masking action of garlic should be allicin, compound first described by Cavallito *et al.* (*7*), which forms from alliin (*8*) by enzyme action of allinase; namely.



Plants of allium species other than garlic, *Allium odorum*, *Allium Bekeri* Regel, for example, were found to be as highly capable of masking effect as garlic, and the resultant compound formed by the masking reaction was shown to be equal to allithiamine in its outstanding rate of permeation through intestinal wall as shown in Table III. There is no doubt that such resultant compound should be allithiamine or its homologues. Considering the mechanism of allithiamine formation as previously shown as a pair of chemical equations, it is highly probable that this kind of plants contains alliin or its homologues. And the masking principles of these plants are probably allicin or its homologues correspondingly.

Previous mention has been made of our proposal relative to the active principles in the natural kingdom which render thiamine negative to thiochrome reaction, that they should be discussed under two different concepts; "inactivation" and "masking action." Our study on a series of similar active principles is in progress. The following is the outline of the information so far available.

Examples of "inactivating factors" are thiaminase and thermostable anti-thiamine factor, and the latter is widely distributed in the plant kingdom.

As the sources of the "masking principles," we have found allium and curciferae in plant, and saliva and gall in animal sources *(9–11)*. In spite of the characteristic feature in common with them that they convert thiamine to a substance which turns back to thiochrome positive

TABLE III

Urinary Excretion of Thiamine after Oral Ingestion of Masked Thiamine by the Allium

	Allium sativum	*Allium odorum*	*Allium Bekeri* Regel	Thiamine
Dose of oral administration	*ca.* 20 mg.	*ca.* 20 mg.	*ca.* 20 mg.	20 mg.
	γ	γ	γ	γ
1 hour after administration	890	780	510	80
2 hours after administration	570	760	480	190
3 hours after administration	450	450	320	150

when reduced, they differ immensely from one another in other aspects of their behavior; for instance, whilst the active principle of allium converts thiamine to allithiamine or its homologues, that of saliva and gall was found to yield thiamine-disulfide. No definite information is avaliable yet as to the active principle of cruciferae species except that the allylmustard oil, a chief constituent of *Brassica cernua* Hemsl. was found to mask and convert thiamine to a compound similar to allithiamine in its ready premeation through intestinal wall. Relative to this aspect of the problem, a further study is being undertaken.

SUMMARY

We have found the fact that when we let an extract of plants of allium species, *e.g.* garlic, react with thiamine, thiamine turns to a hitherto unknown compound which was negative to thiochrome reaction *in vitro*, but acted as thiamine *in vivo*. We have succeeded in isolating this compound and designated it as allithiamine.

Allithiamine has the structure of thiamine allyl disulfide and forms as the resultant compound when allicin reacts with thiamine.

Allithiamine simulates thiamine-disulfide in its biochemical properties, but behaves in a different way from generally accepted behaviors of thiamine or its homologues in that it is highly soluble in organic solvent.

Necessary conditions for quantitative determination of allithiamine have been discussed.

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STUDIES ON THE METABOLISM OF *p*-HYDROXYPHENYLPYRUVIC ACID

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Since 2,5-dihydroxyphenylpyruvic acid was presented by Neubauer (*1*, *2*) as a possible intermediate of L-tyrosine, its metabolic pathway has been investigated by many workers (*3*–*12*) and evidence has also been presented in our laboratory (*13*), using the technique of successive adaptation and liver enzymes, that tyrosine is broken down to homogentisic acid *via p*-phydroxyhenylpyruvic acid and 2,5-dihydroxyphenylpyruvic acid, instead of through 2,5-dihydroxyphenylalanine.

At the present time, the metabolic pathway of L-tyrosine shown in the following scheme is considered to be true; L-tyrosine→*p*-hydroxyphenylpyruvic acid→2,5-dihydroxyphenylpyruvic acid→homogentisic acid→fumarylacetoacetic acid→fumaric acid and acetoacetic acid.

On the basis of the finding that one molecule of L-tyrosine is converted to acetoacetic acid with 4 atoms, instead of 5 calculated theoretically, of O_2-uptake, Felix and Zorn (*14*, *15*) assumed that a transamination reaction may take part in the conversion of L-tyrosine to *p*-hydroxyphenylpyruvic acid. Recently, Knox (*16*) and others (*17*, *18*) have demonstrated the existence of the transamination reaction using rat liver enzymes and also clarified the role of pyridoxal phosphate as a co-enzyme of the reaction. In the same study, Knox (*16*) reported the significance of the action of vitamin C (V. C) on the metabolism of *p*-hydroxyphenylpyruvic acid to homogentisic acid, which was not attributed to the formation of peroxide possibly derived from V. C and was not compensated by such a series of reducing agents as gluta-

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thione, cysteine, and dihydroxymalic acid. In this case, folic acid was found to have no effect on the metabolism.

Authors have investigated the mechanism of the enzymatic oxidation of *p*-hydroxyphenylpyruvic acid and the general properties of the enzyme catalyzing the reaction using purified enzyme preparations obtained from rabbit liver, and have found that the reaction is catalyzed by an oxidase, for which vitamin B_{12} (V. B_{12}) acts as the coenzyme in the presence of V. C. 2,5-Dihydroxyphenylpyruvic acid has been proven to be the intermediate for the oxidation of *p*-hydroxyphenylpyruvic acid using the technique of paperchromatography; the former was also enzymatically converted, after completion of the oxidation of the benzene ring, to homogentisic acid with evolution of carbon dioxide, where cocarboxylase and DPN were involved in the enzymic oxidative decarboxylation of the side chain of the substrate. This communication deals with the investigations described above in the metabolism of *p*-hydroxyphenylpyruvic acid.

EXPERIMENTAL

Materials

Enzyme Preparations—After rabbits weighing 2.5 to 3 kg. were sacrificed by bleeding, the livers were removed, chilled and homogenated with equal amounts of quartz sand in 3 parts of distilled water and were kept for one hour at pH 7.2 (adjusted with 10 per cent Na_2CO_3) in an ice box (0° to 5°). The soluble enzyme was obtained by centrifuging the homogenate at 4000 r.p.m. for 20 minutes and was then subjected to fractionation with saturated ammonium sulfate solution adjusted to pH 7.0 with 10 per cent Na_2CO_3. The 30 to 50 per cent fractions, as shown later, retained the highest activity for the oxidation of *p*-hydroxyphenylpyruvic acid and was adsorbed to 2 per cent calcium phosphate-gel (*19*) for one hour at pH 6.8 and 0° to 5°. The adsorbant was separated from the supernatant at 2000 r.p.m. for 5 minutes and was washed with chilled distilled water until no traces of NH_4^+ remained. In this case, the supernatant obtained by removal of calcium phosphate-gel lost its activity. Subsequently, the absorbant was eluted in 0.2 to 0.1 *M* phosphate buffer (pH 7.8) equal to the volume of the fraction for 3 to 19 hours at 0° to 5°. The supernatant was separated from calcium phosphate-gel by centrifuging at 2000 r.p.m. for 5 minutes.

The eluate thus obtained was used as a purified enzyme.

On the other hand, the crude enzyme treated by isoelectric precipitation was prepared following the method described by Knox (*16*); the pH of rabbit liver extracts described above were adjusted to 5.0 with 0.5 *M* NaH_2PO_4, and was then centrifuged at 4000 r.p.m. for 15 minutes. The supernatant thus obtained was adjusted to pH 7.2 and used for the experiment.

p-Hydroxyphenylpyruvic Acid—*p*-Hydroxyphenylaldehyde, which was prepared from phenol, chloroform and NaOH by the method of Reimer and Tieman (*20*), was condensed with hippuric acid to form its azlactone (*21*), which was decomposed in alkaline reaction according to the procedure of Kotake (*22*): m.p. 215.5°; Millon's and $FeCl_3$ tests were positive.

2,5-*Dihydroxyphenylpyruvic Acid*—This was obtained from the hydrolysis (*23*) of the azlactone which was prepared by the method of Neuberger (*24*): m.p. 225°; Briggs' and hydrazone tests were positive.

Homogentisic Acid—This was isolated as the Pb-salt (m.p. 215°) from ether extracts of the urine of an alcaptonuric. The Pb-slat suspended in peroxide-free ether was treated with H_2S before use, and homogentisic acid was freed; m.p. 147°.

Diphosphopyridine Nucleotide (*DPN*)—This was prepared from brewer's yeast according to the procedure of Williamson (*25*) and purified to 80 per cent by the method of Jandorf (*26*).

Co-carboxylase—This was furnished by the Akabori Laboratory, Osaka University. It was prepared by fusion of vitamin B_1 (V. B_1), pyro- and ortho-phosphates according to the procedure of Tauber (*27*).

Vitamin B_{12} (*V.* B_{12}) *and Folic Acid*—Both were furnished by the Takeda pharmaceutical Co., Ltd.

Glutathione—This was furnished by the Kirin Brewery.

Methods

Determination of O_2*-Uptake*—This was carried out using a conventional Warburg apparatus with the following composition of the vessel contents made up to a final volume of 3.1 ml.

Main compartment: Enzyme solution 1.0 ml., 1 *M* phosphate buffer (pH 7.4) 0.3 ml., and other materials shown in the experiments; finally made up to 2.5 ml. by the addition of distilled water.

Side arm: *p*-Hydroxyphenylpyruvic acid or 2,5-dihydroxyphenylpyruvic acid or homogentisic acid; 1 to 2 micromoles each in 0.5 ml. distilled water.

Center well: 50 per cent KOH 0.1 ml.

The above reaction mixtures were incubated at 37.5°.

Dihydroxyphenyl-derivatives, as reaction products, were determined by a modification of Briggs' test (*28*) and also examined by paperchromatography.

Briggs' Test—To avoid the color presentation in the Briggs' test of *p*-hydroxyphenylpyruvic acid when trichloracetic acid was used as a deproteinizing agent, the reaction mixtures under test were deproteinized by the addition of 1 ml. of 7 *N* H_2SO_4. The filtrate was added to 2 ml. of 1 per cent KH_2PO_4 and 2 ml. of 5 per cent ammonium molybdate in 5 *N* H_2SO_4 and exactly 5 minutes later the color presented was examined. When V. C. was present in the reaction mixture, the filtrate was extracted three times with 10 ml. each of peroxide-free ether and the ether extracts were dissolved in 2 ml. of distilled water following removal of ether. The aqueous layer thus obtained was tested quantitatively according to the procedure of Mazima and Tsuzimoto (*29*), that is, photometrically using a filter S_{72}.

Paperchromatography—The deproteinized filtrate was extracted five times with three times volumes of peroxide-free ether, and the ether extracts were evaporated, and then dissolved in 0.5 ml. of distilled water. The aqueous solution thus obtained was used for paperchromatography. The developing solvent was a mixture of *n*-butanol, glacial acetic acid and distilled water (4 : 1 : 1). Spots developed on the filter paper were tested by spray of 10 per cent ammoniacal $AgNO_3$ solution and of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 *N* HCl.

Determination on Acetoacetic Acid—This was carried out according to the aniline citrate method of Edson (*30*).

RESULTS

I. *Experiments with Crude Enzymes*

Activity of Rabbit Liver Extract—*p*-Hydroxyphenylpyruvic acid was decomposed by 91 per cent of the theoretical amounts by liver extract, while L-tyrosine decomposed by 17 per cent.

Optimal pH—Since the decomposition of *p*-hydroxyphenylpyruvic acid was maximum at pH 7.0 to 8.0, subsequent experiments were carried out at pH 7.4, unless otherwise specified.

Quantity of Substrate—More than 2 micromoles of *p*-hydroxyphenylpyruvic acid was found to decrease the rate of decomposition of the substrate, using either the liver extract or the crude enzyme obtained by isoelectric precipitation. Therefore, subsequent experiments were carried out with the quantity of the substrate ranging from 1 to 2 micromoles.

Fractionation with $(NH_4)_2SO_4$ and Isoelectric Precipitation—As shown in Table I, the maximum activity of the enzymes fractioned with $(NH_4)_2SO_4$ was found to lie in the 30 to 50 per cent fractions. The 0 to 30 per cent fractions showed only 30 per cent of the activity of the liver extract, while the over 50 per cent fractions lost the activity completely. As a whole, the enzymatic activity decreased nearly 18 per cent, after fractionation, as compared with that of the liver extract. Moreover, the liver extract lost, after standing of 24 hours, 35 per cent is activity which had been found when freshly prepared: the 30 to 50 per cent fractions lost 75 per cent its activity under the same conditions.

According to calculation on the basis of acetoacetic acid production, the amount of *p*-hydroxyphenylpyruvic acid decomposed was in general approximately 20 per cent lower than that calculated on the basis of O_2-uptake. Considering the disappearance of Briggs' test in each case, the above difference may account for the involving of O_2-uptake due to the autoxidation of some intermediates, *i. e.* dihydroxyphenyl-derivatives, in the metabolic process between *p*-hydroxyphenylpyruvic acid and homogentisic acid.

While the supernatant (I) obtained by removal of the residue after isoelectric precipitation of the liver extract decomposed *p*-hydroxyphenylpyruvic acid with 1.9 atoms of O_2-uptake, the residue fraction (II) showed only 0.18 atoms of O_2-uptake. On the

TABLE I

O_2-Uptake and Acetoacetic Acid Production by Each Fraction

The contents of the vessels were made up as described in the method (a), except for enzyme preparations and the substrates added to each of the runs. *p*-Hydroxyphenylpyruvic acid: 2 micromoles. Each of the fractions was prepared by dissolving the precipitates fractionated with ammonium sulfate in distilled water so as to make the rate of dilution equal to that of the liver extract.

		Liver extract	$(NH_4)_2SO_4$ fractions		
			The 30 to 50%	The 0 to 30%	The 50 to 100%
O_2-uptake	*atoms*	3.7	3.0	1.1	0
Briggs' test		—	—	—	—
Acetoacetic acid	*molecules*	0.78	0.61	0.23	0

other hand, neither of the fractions oxidized homogentisic acid. The 30 to 50 per cent fraction obtained by fractionating the supernatant (I) with ammonium sulfate retained the highest activity. Both the supernatant and the 30 to 50 per cent fraction lost their activities completely after standing 24 hours. Briggs' test was positive for the incubated reaction mixtures (I), (II).

Production of CO_2—p-Hydroxyphenylpyruvic acid was oxidized by the 30 to 50 per cent fraction obtained from the supernatant which was prepared by isoelectric precipitation of the liver extract in the same manner as in the previous paragraph, with 1.62 atoms of O_2-uptake and the evolution of 0.6 molecules of CO_2 (74 per cent of the theoretical values calculated from the O_2-uptake).

The evolution of CO_2 was hardly recognized until 1 atom of oxygen was consumed. Briggs' test for the reaction mixture, after incubation, was also positive.

Behavior of the Inhibitors—The activity of the enzyme separated by isoelectric precipitation from the system oxidizing homogentisic acid was completely inhibited by oxin and H_2S; 79.5 per cent by NaN_3 partially by monoiodoacetic acid. KCN, α,α'-dipyridyl and CO had almost no inhibitory effect (Table II).

Activation of the 30 *to* 50 *per cent Fraction Inhibited by Oxin*—While the 30 to 50 per cent fraction was completely inhibited by oxin, its activity was restored with respect to the O_2-uptake for *p*-hydroxyphenylpyruvic acid by the addition of V.B_{12} or/and V.C, or methylene blue. In this case, folic acid had no effect. In the case of resumption of the O_2-uptake by the above additions, the formation of dihydroxphenyl-derivatives in the reaction mixtures was confirmed by Briggs' test of their ether extracts.

Production of H_2O_2—As shown in Table III, the O_2-uptake for *p*-hydroxyphenylpyruvic acid was suppressed by the addition of ethanol to the same extent as the O_2-uptake due to endogeneous respiration. Consequently, no hydrogen peroxide was

TABLE II

Behaviors of a Variety of Inhibitors on the Enzyme Activity

1 ml. of the crude enzyme, prepared by isoelectric precipitation of the liver extract; the final concentration of each inhibitor was made up as shown in the Table. The experiments for H_2S and CO were carried out using the enzyme preparations through which either of the gases was preliminarily bubbled for 5 to 15 minutes. Other experimental conditions were the same as in Table I.

Concentration	Inhibitors	Percentage of inhibition*
M		*per cent*
10^{-3}	Oxin	100
	H_2S	100
10^{-3}	NaN_3	79.5
10^{-3}	Monoiodoacetic acid	38.4
10^{-3}	Urethane	18.6†
10^{-3}	α,α'-Dipyridyl	15.4
10^{-3}	Benzoic acid	10.9
10^{-3}	Mercurochloro benzoate	10.9
10^{-3}	$HgCl_2$	10.8
10^{-3}	NaF	10.2
10^{-3}	$Na_2S_2O_3$	10.2
10^{-2}	KCN	8.1
10^{-3}	KCN	5.3
10^{-3}	Sulfamine	0
10^{-3}	KCNS	0
	CO	0

* Calculated on the basis of the O_2-uptake for the oxidation of *p*-hydroxy phenylpyruvic acid during incubation for 60 minutes.

† The inhibitor was added to the enzyme up to the indicated concentration and kept at room temperature for one hour and the experiment was then carried out using this preparation.

conceivably produced in the metabolic process between *p*-hydroxyphenylpyruvic acid and homogentisic acid.

II. Experiments with Purified Enzymes

Effect of Various Compounds on Activation of the Enzyme—The eluate prepared according to the procedure in the section of materials showed little or no activity for the oxidation of *p*-hydroxyphenylpyruvic acid as illustrated in Figs. 2 and 4. Although either the increase or the resumption of the O_2-uptake effected by adding some substances does not necessarily account for the restoration of the activity of the eluate for

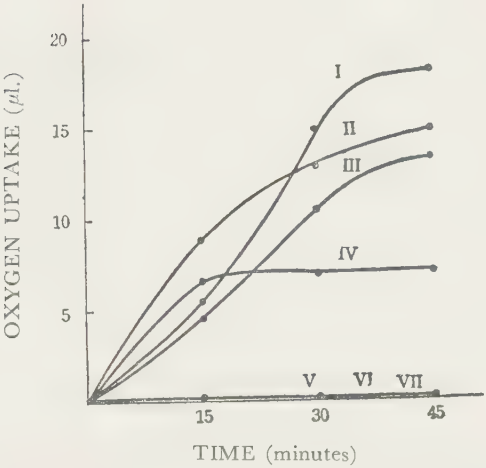

FIG. 1. Activation of the 30 to 50 per cent fraction inhibited by Oxin.

The experimental conditions were the same as in Table I, except for 1 ml. of the 30 to 50 per cent fraction treated in the following way and the compounds added (I to VI). The fraction obtained from the liver extract as in the paragraph (d) was preliminarily inhibited by adding oxin to the final concentration of 10^{-3} *M* and was then removed from the surplus amount of inhibitor remaining in the medium by repeated fractionation of the same fraction at the level of 50 per cent saturation with ammonium sulfate. The concentration of oxin in the fraction was finally reduced to nearly 10^{-7} *M*.

I. V.B_{12}, 80 μg. plus V. C, 500 μg. II. V. C, 500 μg. III. 2×10^{-4} *M* methylene blue, 0.3 ml. IV. V.B_{12}, 80 μg. V. Folic acid, 500 μg. VI. 10^{-2} *M* $FeSO_4$, 0.3 ml. VII. No addition (control).

the reasons mentioned below, such compounds that affect the O_2-uptake for *p*-hydroxyphenylpyruvic acid are shown in Table IV. The O_2-uptake was increased by the addition of the following compounds under the experimental conditions: methylene blue, V. C., V. C plus V. B_{12}, V. C plus folic acid, V. C plus Fe^{++}, and 5-hydroxyanthranilic acid; while bivalent metal ions had no effect.

Behavior of V.B_{12} and V.C on the Decomposition of p-Hydroxyphenylpyruvic Acid—As shown in Fig. 2, V.B_{12} showed an inhibitory action in a single addition, on the eluate which decomposed *p*-hydroxyphenylpyruvic acid with 0.41 atoms of O_2-uptake, while it increased more markedly the activity of the eluate in combination with V.C than did V. C alone. In the case of the combination of V. B_{12} and V. C the ether extracts of the reaction mixture gave an intensive Briggs' test to indicate the formation of di-

TABLE III

Influence of Ethanol on the Oxidation of p-Hydroxyphenylpyruvic Acid

1 ml. of the supernatant obtained by isoelectric precipitation of the liver extract was placed in each of the vessels from I to IV:

I. No addition.
II. Ethanol, 2 micromoles.
III. *p*-Hydroxyphenylpyruvic acid, 2 micromoles.
IV. *p*-Hydroxyphenylpyruvic acid, 2 micromoles plus ethanol, 2 micromoles.

O_2-uptake of each was determined for 45 minutes after tipping the substrates into main compartment. Other experimental conditions were the same as in Table I.

	Vessels			
	I	II	III	IV
O_2-uptake in 45 minutes after tipping *μl.*	22.5	19.5	60.2	57.6

hydroxyphenyl-derivatives as intermediates along with increase of O_2-uptake; whereas the enhancement of O_2-uptake on adding V. C was found not to be derived from the increased rate of the decomposition of the substrate, for the intensity of the Briggs' test for the ether extracts of the reaction mixture in which V. C alone had been added was the same as that for the ether extracts of the reaction mixture without its addition.

On the other hand, V.C alone did not effect the restoration of activity of the eluate or the resumption of O_2-uptake when the enzyme was completely inactivated, while $V.B_{12}$ and other compounds, as shown below in Fig. 4, restored its activity. As discussed later, the effect of V.C alone on increasing the O_2-uptake for *p*-hydroxyphenylpyruvic acid with the eluate of poor activity is conceivably attributed to the increasing rate of autoxidation of V.C itself caused by catalytic action of the dihydroxyphenyl-derivative which has been formed by the action of the eluate alone on the substrate.

Ability of Eluate to Decompose Homogentisic Acid in the Presence of V.C—About 2 atoms of oxygen, as shown in Fig. 3, were consumed at the first stage (I), whereas no acetoacetic acid was formed. At the second stage (II) where the liver extract was newly added to the above reaction mixture, 2 atoms of additional O_2-uptake occurred with the simultaneous formation of the theoretical amount (1 molecule) of acetoacetic acid. From these data, the occurrence of O_2-uptake at the first stage was interpreted by an increased rate of autoxidation of V. C catalyzed by homogentisic acid, which had not been oxidized by the eluate at all.

Behavior of $V.B_{12}$, V.C, Adenylic Acid, Glutathione, Co-carboxylase and DPN on the Enzyme Activity—The completely inactivated eluate regained its activity with 0.4 atoms of O_2-uptake by the simultaneous addition of glutathione, adenylic acid, co-carboxylase and DPN in the presence of methylene blue. When $V.B_{12}$ was supplied in addition to the

TABLE IV

Effect of Various Compounds added to the Eluate on O_2-Uptake

Each of the compounds to be added to the eluate were dissolved in distilled water and were made up to the indicated concentration in the table. Each of the vessels contained 1 ml. of the eluate and 1 micromole of *p*-hydroxyphenylpyruvic acid.

Incubation time: 75 to 90 minutes. Other experimental conditions were the same as in Table I. The O_2-uptake of each of the runs was compared with that of the eluate without adding.

Compound added	Effect on O_2-uptake*
$FeSO_4$ (10^{-3} *M*)	—
$CoCl_2$ (10^{-3} *M*)	—
$CuSO_4$ (10^{-3} *M*)	—
$H_2S_2O_4$ (10^{-3} *M*)	—
V.B_{12} (20 μg.)	—
DPN (50 μg.)	—
V.C (500 μg.)	+
Folic acid (50 μg.)	—
Glutathion (1000 μg.)	—
Co-carboxylase (50 μg.)	—
Methylene blue (2×10^{-5} *M*)	+
5-Hydroxyanthranilic acid (10^{-3} *M*)	+
V.B_{12} (20 μg.) and V.C (500 μg.)	+
V.B_{12} (20 μg.) and $H_2S_2O_4$ (10^{-3} *M*)	—
V.B_{12} (20 μg.) and 5-hydroxyanthranilic acid (10^{-3} *M*)	+
Folic acid (500 μg.) and V.C (500 μg.)	+
$FeSO_4$ (10^{-3} *M*) and V.C (500 μg.)	+
Co-carboxylase (50 μg.) and DPN (50 μg.)	—
Methylene blue (2×10^{-5} *M*) and adenylic acid (500 μg.)	—
Methylene blue (2×10^{-5}*M*), adenylic acid (500 μg.), co-carboxylase (50 μg.) and DPN (50 μg.)	+
Methylene blue (2×10^{-5}*M*), adenylic acid (500 μg.), co-carboxylase (50μg.), DPN (50 μg.) and V.B_{12} (20 μg.)	+

* (+) Indicates the increase of O_2-uptake. (—) No effect on O_2-uptake.

above four compounds, the enzyme activity was more markedly restored with 1.2 atoms of O_2-uptake and the production of 0.26 molecules (87 per cent of the theoretical amounts calculated on the basis of the corresponding O_2-uptake) of acetoacetic acid (Fig. 4).

When the activity of the eluate for *p*-hydroxyphenylpyruvic acid oxidation was restored by the above four compounds and V. C in the presence of methylene blue, the evolution of CO_2 of 0.15 molecules was observed for 1 atom of O_2-uptake during the oxidation process of the substrate (Fig. 5). In this case, acetoacetic acid was not found in the reaction mixture.

The discrepancy in the results between Figs. 4 and 5 with respect to acetoacetic acid formation by the reactivated enzymes as shown above may be explained by the evidence (*31*) that fumarylacetoacetic acid hydrolylase (*32*) which links up with the system decomposing homogentisic acid, is liable to be removed from the enzyme preparation at this stage of purification.

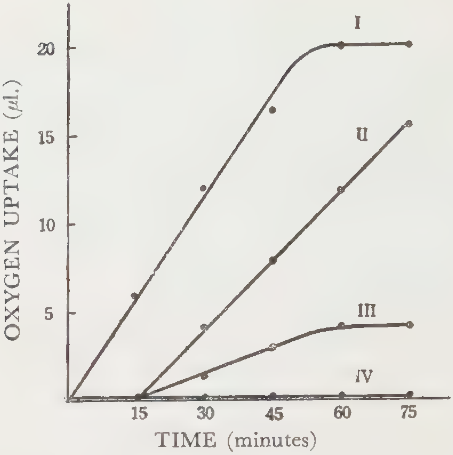

FIG. 2. Effect of V. B_{12} and V. C on the decomposition of *p*-hydroxyphenylpyruvic acid with the eluate.

V.B_{12} and V.C were added respectively to each of the runs (I to VI).

I. V.B_{12}, 20 μg. plus V.C, 500 μg. II. V.C, 500 μg. III. No addition. IV. V.B_{12}, 20 μg.

Other experimental conditions were the same as in Table IV.

Effect of Folic Acid on the Enzyme Activity—Folic acid completely inhibited the enzyme in a single addition (IV), but increased the O_2-uptake in combination with V.C (II) to a lesser extent than did V.C alone (I). The intensity of Briggs' test for the reaction mixture supplemented either with folic acid and V.C or V.C alone was almost the same as that of the control run (III). Therefore, the increase of the O_2-uptake induced by folic acid and V.C may be explained as being derived from the increased rate of the autoxidation of V.C, as shown in Figs. 2 and 3 (Fig. 6).

Effect of Bivalent Ions on the Enzyme Activity—Fe^{++} completely inhibited the enzyme in a single addition (IV), but increased the O_2-uptake markedly in combination with V.C under the experimental conditions (I), where the intensity of Briggs' test for its reaction mixture was the same as that of the control run (II) (Fig. 7).

The activity of the eluate enhanced by adding V.B_{12}, V.C, adenylic acid, glutathione, co-carboxylase and DPN as shown in Fig. 4 was also inhibited by Fe^{++} (Fig. 8). The fact is in accord with the inhibitory action of Fe^{++} on the purified system oxidizing homogentisic acid and on the increased rate of the autoxidation of V.C catalyzed by homogentisic acid reported by Uchida and Uematsu (*31*).

As shown in Fig. 15, both Cu^{++} and Co^{++} had little effect on enzyme activity.

Saturation Curve of V. B_{12} for the Restoration of the Activity of the Resolved Purified Enzyme in the Presence of V. C—It was found that 20 μg. of V. B_{12} was required for

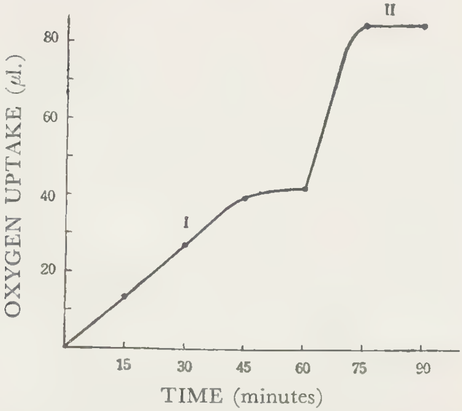

Fig. 3. Oxidation of homogentisic acid by the eluate in the presence of V. C.

The experimental conditions were the same as in Fig. 2, except for the following compounds.

I. Homogentisic acid, 2 micromoles; V. C., 500 μg. II. 1 ml. of freshly prepared liver extract as described in the section of materials was placed in a side arm of each of the vessels including the control run and added after the O_2-uptake attained to an saturation level at the first stage (I).

a maximum restoration of the activity of the eluate which had been completely inactivated, by determining the rate of increase of the *p*-hydroxyphenylpyruvic acid oxidation in a variety of the concentrations of V. B_{12} ranging from 5 to 50 μg. (Fig. 10).

On the basis of the determination of dihydroxyphenyl-derivatives formed as the reaction products, V. B_{12} requirements for the same maximum restoration of the enzyme activity was established to be 15 μg. (Table V), where 72 per cent of the initial amounts of *p*-hydroxyphenylpyruvic acid were oxidatively converted to dihydroxyphenyl-derivatives by the restored enzyme.

Investigations on the Reaction Products by Paperchromatography—While, only the spot of *p*-hydroxyphenylpyruvic acid (Rf, 0.88) was found in the run I, as shown in Fig. 11 (a), two different spots (Rf, 0.92, 0.52) which coincide with those of the authentic sample of 2,5-dihydroxyphenylpyruvic acid (Rf, 0.90, 0.52) as shown in Fig. 16 (a), were developed, on the filter paper in the runs supplemented with either V. C (II) or V. B_{12} plus V. C (III). The color presented by 2,4-dinitrophenylhydrazine test for these spots was more intense in the latter supplementation than in the former. In each of the runs II, III, moreover, a trace of homogentisic acid was recognized, developing on the filter paper as a spot with the Rf value of 0.72.

When the eluate was supplemented with DPN, co-carboxylase and methylene

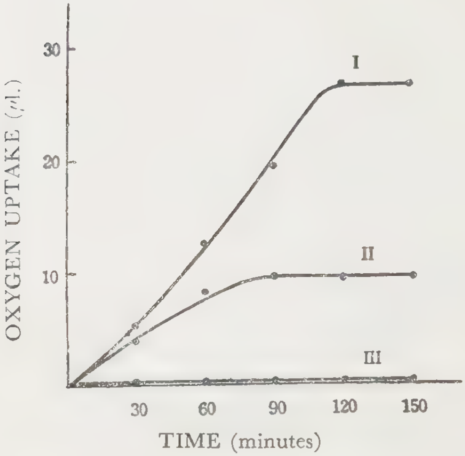

Fig. 4. Effect of V.B_{12}, adenylic acid, glutathione, co-carboxylase and DPN on the activity of the eluate.

The experimental conditions were the same as in Fig. 2, except for 2 micromoles of *p*-hydroxyphenylpyruvic acid and other compounds being added to each of the runs (I, II).

I. V. B_{12}, 15 μg.; adenylic acid, 500 μg.; glutathione, 1 mg.; co-carboxylase, 50 μg.; (2×10^{-4} *M.*) methylene blue, 0.3 ml. II. V. B_{12} was removed from I. III. No addition (control).

blue, in addition to V.B_{12} and V.C, the paperchromatograms of the reaction mixture, contrary to the previous experiment, showed the spot of homogentisic acid much more distinctly with the Rf value of 0.72 (Fig. 12 (b)). Meanwhile, the spots of 2,5-dihydroxyphenylpyruvic acid that had been previously noticed almost disappeared and only a trace was found remaining as a spot with the Rf value of 0.29 (Fig. 12 (a)) as shown in Fig. 22 (b).

III. Enzymic Conversion of 2,5-Dihydroxyphenylpyruvic Acid to Homogentisic Acid-Oxidative Decarboxylation of 2,5-Dihydroxyphenylpyruvic Acid with the Purified Enzyme

The eluate, the same as in the previous experiments, which had been inactivated, regained its activity by supplement of co-carboxylase and DPN, and gave rise to 0.4 atoms of O_2-uptake in the oxidation of 2,5-dihydroxyphenylpyruvic acid (Fig. 13 (III)). The additional supplement of methylene blue, besides the above two compounds, resulted in approximately 1 atom of O_2-uptake (Fig. 13 (II)) and simultaneously the evolution of 61 per cent the theoretical amounts of CO_2 (Fig. 14).

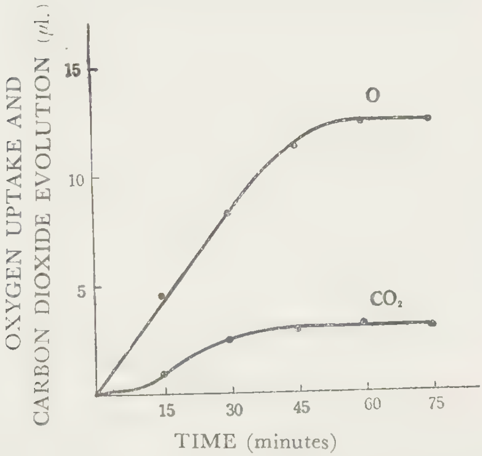

Fig. 5. Relation between CO_2 production and O_2-uptake in the decomposition of *p*-hydroxyphenylpyruvic Acid.

I. V. C (500 μg.) was added at the same time to the compounds mentioned in Fig. 4 (I). II. The center wells of the vessels for the determination of CO_2 were devoid of KOH solution. Other experimental conditions were the same as in Fig. 4.

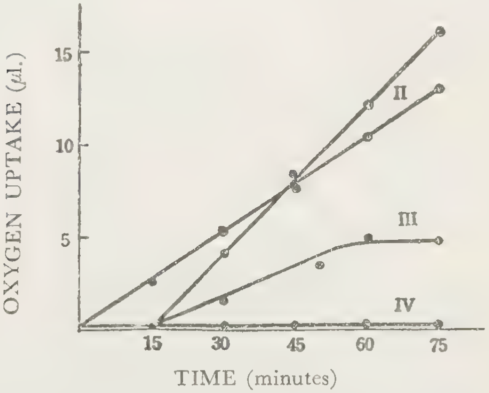

Fig. 6. Effect of folic acid on the enzyme activity.

The experimental conditions were the same as in Fig. 2, except for the compounds added to each of the runs (I to IV).

I. V. C, 500 μg. II. Folic acid, 500 μg. plus V. C, 500 μg.
III. No addition (control). IV. Folic acid, 500 μg.

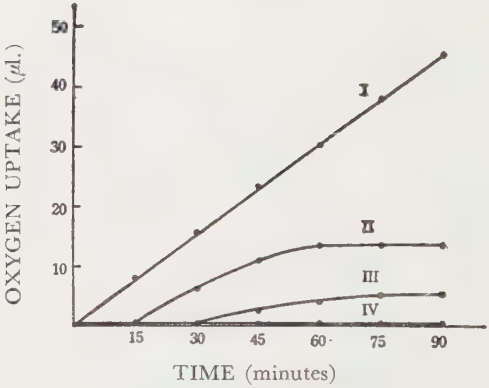

FIG. 7. Effect of Fe^{++} and V. C on the enzyme activity.

The experimental conditions were the same as in Fig. 2, except for the compounds added to each of the runs (I to IV).

I. V. C, 500 μg. plus 10^{-2} *M* $FeSO_4$, 3 ml. II. V. C, 500 μg., III. No addition (control). IV. 10^{-2} *M* $FeSO_4$, 0.3 ml.

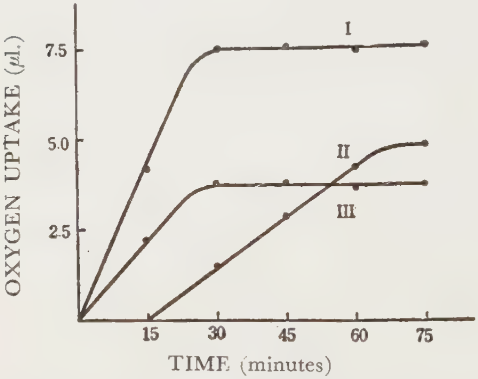

FIG. 8. Effect of Fe^{++} on the enzyme activity.

I. The experimental conditions were the same as in Fig. 5 (I). II. The same as in I, except for the additional supplementation of 0.3 ml. of 10^{-2} *M* $FeSO_4$ to the main compartments of the vessels. III. The same as in Fig. 5 (III) (control).

The remarkable increase of the O_2-uptake found after the single addition of methylene blue (Fig. 13 (I)) was attributed mainly to its capacity to accelerate the autoxidation of the substrate, as shown in Fig. 15.

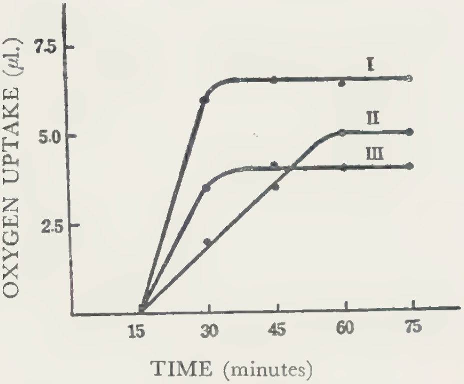

FIG. 9. Effect of Cu^{++} and Co^{++} on the enzyme activity.

The experimental conditions were the same as in Fig. 2, except for the compounds added to each of the runs (I to III).

I. 10^{-2} *M* $CuSO_4$, 0.3 ml. II. No addition (control).

III. 10^{-2} *M* $CoCl_2$, 0.3 ml.

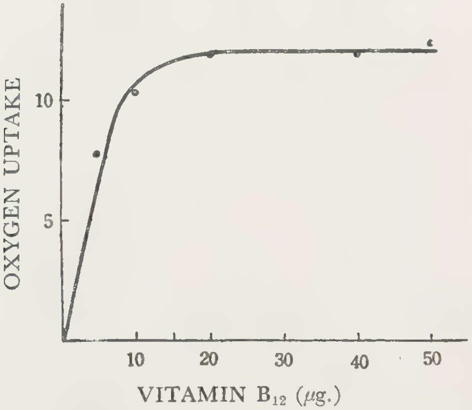

FIG. 10. Saturation curve of V. B_{12} for the oxidation of *p*-hydroxyphenylpyruvic aicid with the resolved enzyme.

The experimental conditions were the same as in Fig. 2, except for a variety of the amounts of V. B_{12} ranging from 5 to 50 μg. as indicated in the table.

2,5-Dihydroxyphenylpyruvic acid, as shown in Fig. 15, was readily autoxidized with nearly 0.7 atoms of O_2-uptake at pH 7.4, where the rate of autoxidation was

TABLE V

Relation between the Amounts of V. B_{12} Restoring Resolved Enzyme Activity and Dihydroxyphenyl Derivatives Formation

The determination of dihydroxyphenyl-derivatives were carried out by a modified Briggs' test, as shown in the section of methods, for the ether extracts of the reaction mixtures. Other experimental conditions were the same as in Fig. 10.

	V. B_{12} (μg.)						
	5	10	15	20	30	40	50
Dihydroxy derivatives formation μg.	89.1	118.6	130.2	125.7	—	123.0	142.9

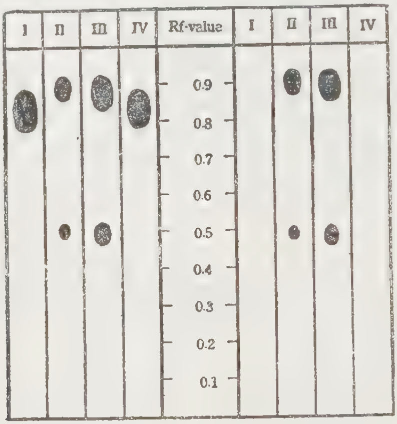

FIG. 11 (a) 2,4-Dinitrophenylhydrazine

FIG. 11 (b) Ammoniacal $AgNO_3$

FIG. 11. (a) (b). Paperchromatograms of the reaction products with the eluate supplemented with V. B_{12} and V. C.

The contents of the reaction mixtures were prepared as follows: the eluate 20 ml., 1 *M* phosphate buffer (pH 7.4) 6.0 ml., *p*-phydroxyphenylpyruvic acid 7.2 mg. in 10 ml. of distilled water and the compounds as shown below were combined together and made up to a total volume of 60 ml. by adding distilled water.

I. No addition (control). II. V. C, 10 mg. III. V. B_{12}, 300 μg. plus V. C, 10 mg. IV. Authentic sample of *p*-hydroxyphenylpyruvic acid dissolved in distilled water was provided for paperchromatography.

The reaction mixtures were deproteinized after incubation of 4 hours at 37.5° and the ether extracts were investigated by paperchromatography as mentioned in the section of methods, using either 2,4-dinitrophenylhydrazine-test (Fig. 11 (a)) or ammoniacal $AgNO_3$ solution (Fig. 11 (b)) for the identification of the spots developed.

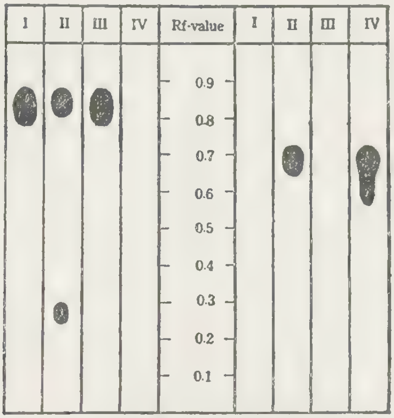

FIG. 12 (a)
2,4-Dinitrophenylhydrazine

FIG. 12 (b)
Ammoniacal
$AgNO_3$

FIG. 12 (a) (b). Paperchromatograms of the reaction products obtained with the eluate supplemented with DPN, co-carboxylase and methylene blue in addition to V. B_{12} and V. C.

The experimental conditions were the same as in Fig. 11 (a) (b), except for the contents of the reaction mixtures.

I. Eluate, 35 ml.; *p*-hydroxyphenylpyruvic acid, 12.6 mg. II. Eluate, 35 ml.; *p*-hydroxyphenylpyruvic acid, 12.6 mg.; V. B_{12}, 600 μg.; V. C, 175 mg.; co-carboxylase, 3.5 mg.; DPN, 3.5 mg.; 2×10^{-4} *M* methylene blue, 4 ml. III. Authentic sample of *p*-hydroxyphenylpyruvic acid dissolved in distilled water. IV. Pure sample of homogentisic acid.

Each of the above runs I, II was made up to a total volume of 105 ml. by adding 10.5 ml. of 1 *M* phosphate buffer (pH 7.4) and distilled water.

increased by 61 per cent (1,2 atoms of O_2-uptake) in the presence of methylene blue.

Investigations of the Reation Products by Paperchromatography—When the substrate was incubated with only the eluate, the paperchromatogram of the reaction mixture (I) showed three spots (Rf, 0.90, 0.50, 0.29) originally pertaining to 2,5-dihydroxyphenylpyruvic acid; whereas the supplement of DPN, co-carboxylase and methylene blue to the eluate (III) resulted in the formation of homogentisic acid which was identified as a spot with the Rf value of 0.81 and remarkable reducing ability. Methylene blue, in a single addition, caused the formation of a trace of homogentisic acid. This fact probably accounts for its slight activation of the eluate during such a long duration of incubation in the experiment (Fig. 16).

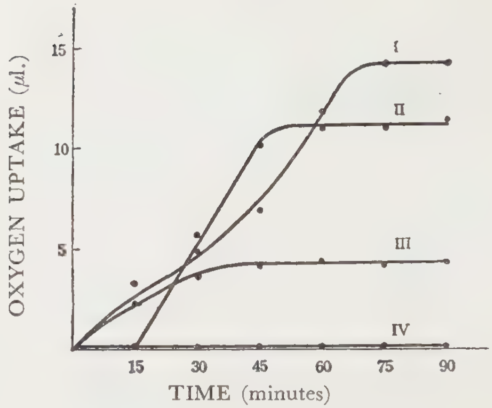

Fig. 13. O_2-uptake of 2,5-dihydroxyphenylpyruvic acid with the eluate in the presence of DPN, co-carboxylase and methylene blue.

The experimental conditions were the same as in Fig. 2, except for the compounds added to the eluate.

I. $2\times10^{-4}\,M$ methylene blue, 0.3 ml. II. Co-carboxylase, 200 μg.; DPN, 100 μg.; $2\times10^{-4}\,M$ methylene blue, 0.2 ml. III. Co-carboxylase, 200 μg.; DPN, 100 μg. IV. No addition.

2, 5-Dihydroxyphenylpyruvic acid, 1 micromole.

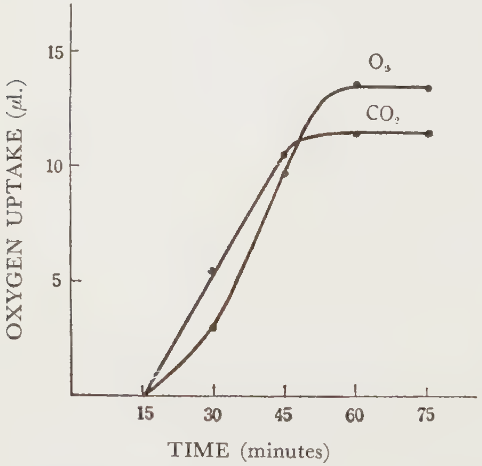

Fig. 14. Relation between O_2-uptake and CO_2 evolution in the oxidation of 2,5-dihydroxyphenylpyruvic acid.

The experimental conditions were the same as in Fig. 13 (II). The center wells of the vessels for the determination of CO_2 were devoid of KOH solution.

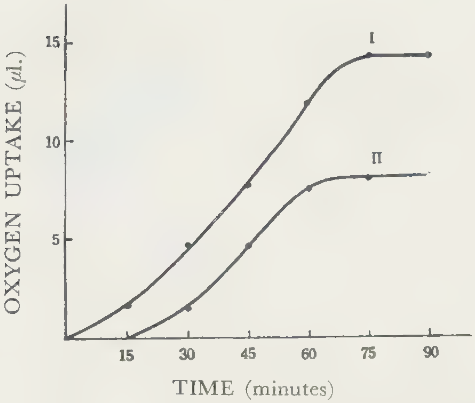

FIG. 15. Autoxidation of 2,5-dihydroxyphenylpyruvic acid and its acceleration by methylene blue.

The experimental conditions were the same as in Fig. 2, except for the addition of methylene blue and the replacement of the eluate with an equal amount of distilled water.

I. 2×10^{-4} *M* methylene blue, 0.3 ml. II. No addition.

2,5-Dihydroxyphenylpyruvic acid, 1 micromole.

DISCUSSION

Viewed from the above data concerning the restoration of activity of the resolved enzymes, the system prepared from the rabbit liver that oxidizes *p*-hydroxyphenylpyruvic acid to homogentisic acid is obviously soluble, excluding mitochondrial structures.

On the basis of the inhibitory action of complex-formers on the enzyme, such as oxin, H_2S and NaN_3, the reactive group responsible for the oxidation of *p*-hydroxyphenylpyruvic acid is considered to be a metal compound, however, the behavior of KCN (Table II) suggests that the adduction of the cyano-group to the metal component, if possible, has no influence on the activity. On the other hand, Fe^{++}, Co^{++} and Cu^{++} have been excluded from the possibility of taking part in the enzyme action (Figs. 1, 7, 8 and 9).

In light of the findings that although V. C alone has no effect on the restoration of the resolved enzyme (Fig. 10), the additional supplement of V. B_{12} initiates the recovery of the enzyme activity to give rise to the formation of dihydroxyphenyl-derivatives (Fig. 10, Table V) and that 2,5-dihydroxyphenylpyruvic acid is demonstrated as the me-

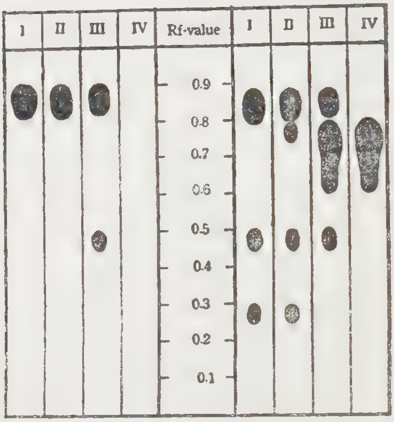

Fig. 16 (a) 2,4-Dinitrophenylhydrazine

Fig. 16 (b) Ammoniacal $AgNO_3$

Fig. 16. Paperchromatograms of the reaction products with the eluate supplemented with DPN, co-carboxylase and methylene blue. The experimental conditions were the same as in Fig. 11, except for the compounds added.

I. Eluate, 20 ml.; 2,5-dihydroxyphenylpyruvic acid, 7.8 mg. II. Eluate, 20 ml.; 2,5-dihydroxyphenylpyruvic acid, 7.8 mg.; $2\times 10^{-4}\,M$ methylene blue, 2 ml. III. Eluate, 20 ml.; 2,5-dihydroxyphenylpyruvic acid, 7.8 mg.; co-carboxylase, 2 mg.; DPN, 2 mg. IV. Pure sample of homogentisic acid dissolved in distilled water (control).

tabolite in oxidizing *p*-hydroxyphenylpyruvic acid with the resolved enzyme that has regained its activity in the combination of V. B_{12} and V. C (Fig. 11), there is no doubt that, in the presence of V. C, V. B_{12} operates in the oxidative conversion of *p*-hydroxyphenylpyruvic acid to 2,5-dihydroxyphenylpyruvic acid.

In support of the fact, evidence has been presented that the enzyme that regained its activity by V. B_{12} in the presence of reducing agents, oxidized the substrate to acetoacetic acid with the evolution of CO_2 when linked up with a sequence of enzymes including the homogentisic acid oxidizing system (Figs. 4 and 5).

In addition to the above results, the fact that the quantity of V. B_{12} required for a maximum rate of restoring the inactivated enzyme attains a saturation level ranging from 15 to 20 μg. points out that the vitamin acts as the co-enzyme for the enzyme oxidizing *p*-hydroxyphenylpyruvic acid to 2,5-dihydroxyphenylpyruvic acid when V. C is present as demonstrated with such systems oxidizing homogentisic acid (*31*), indole (*32*) and catechole (*33*).

Schieve (*34*), Welch *et al.* (*35*) and others (*36*, *37*, *38*, *39*) reported the close relationship between V. B_{12} and reducing agents, especially V. C in growth experiments using microorganisms. Moreover, according to the evidences regarding the increasing stability (*40*) and the removal of the cyano-group (*41*) of V. B_{12} under the influence of reducing agents, it is tenable to consider that the action of V. C on the enzymes shown above may be concerned with stabilizing the reactive form of V. B_{12}, which being suggested to be aquocobalamine hydroxide, instead of its cyano-form, bound to the protein moiety of the enzyme as reported by Cooley *et al.* (*42*) and others (*43*, *44*, *45*, *46*).

From the results, besides those above, relating to the inefficiency of V. C in a single addition and to the exclusion of peroxide formation in oxidizing *p*-hydroxyphenylpyruvic acid (Table III), V. C obviously does not serve as a source of peroxide in the reaction and it can be assumed that the enzyme oxidizing *p*-hydroxyphenylpyruvic acid to 2,5-dihydroxyphenylpyruvic acid is an oxidase of the nature.

The acceleration of O_2-uptake, but not of the enzyme activity, observed in the precence of V. C with the purified enzyme (Fig. 2 and 3) can be attributed to the increased rate of autoxidation of V. C catalyzed either by 2,5-dihydroxyphenylpyruvic acid or homogentisic acid which are the reaction products. In this connection, the enhanced autoxidation of V. C of this type does not occur with crude enzyme preparations and, when denatured, even with purified enzymes.

The fact that the enzyme preliminarily inactivated by oxin regains its activity either by V. C or methylene blue (Fig. 1) coincides with the efficacy of reducing agents observed in removing the inhibitory actions of oxin and NaN_3 on the systems oxidizing homogentisic acid and catechole, which influence, owing to a possible change in the complex formed, being under investigation.

Although it has been reported by Woodruff and Darby (*47*) that the metabolic rate of L-tyrosine in the scorbutic guinea pig is increased by folic acid, its inhibitory effect shown in Figs. 1 and 6 denies the possibility for this compound to operate in the oxidation process of *p*-hydroxyphenylpyruvic acid to homogentisic acid. Nevertheless, it has been confirmed by Uchida and Uematsu (*31*) that folic acid is operative in the system oxidizing homogentisic acid.

In addition, the adsorption spectrum of the eluate inactivated shows two maximum peaks at the wave lengths of 270 and 340 mμ, respectively, instead of those specific for V. B_{12}.

As shown in Figs. 13 and 14, the fact that the resolved purified enzyme when supplemented with DPN and co-carboxylase, in the presence of methylene blue, is capable of oxidizing 2,5-dihydroxyphenylpyruvic acid with 1 atom of O_2-uptake and the evolution of CO_2 of 0.61 molecules, is in accord with the observations on tracing the reaction products by paperchromatography (Figs. 11, 12 and 16) that 2,5-dihydroxyphenylpyruvic acid which has been converted from *p*-hydroxyphenylpyruvic acid with the purified enzyme restored by supplementing with V. B_{12} and V. C is further decomposed to homogentisic acid when the subsequent enzyme systems are linked up with the former by the supply of DPN and co-carboxylase. Consequently, it can be claimed that 2,5-dihydroxyphenylpyruvic acid as the normal metabolite of *p*-hydroxyphenylpyruvic acid undergoes oxidative decarboxylation to form homogentisic acid, where DPN and co-carboxylase are both concerned with the reaction. In this case, the reaction velocity seems more rapid with methylene blue as a hydrogen acceptor than with oxygen (Fig. 15), despite the incidence of the enhancement of autoxidation of the substrate and homogentisic acid (Figs. 15 and 16).

With respect to the relation between the enzyme activity and the concentration of *p*-hydroxyphenylpyruvic acid, the latter ranging over more than 2 micromoles interferes with the former inversely proportional to increasing amounts.

On the other hand, the evolution of CO_2 is hardly found in the decomposition of *p*-hydroxyphenylpyruvic acid with the crude enzyme that involves the system capable of converting the substrate to homogentisic acid, until *p*-hydroxyphenylpyruvic acid undertakes the enzymic oxidation with 1 atom of O_2-uptake. Considering the above results, there is no doubt that the oxidation of *p*-hydroxyphenylpyruvic acid to 2,5-dihydroxyphenylpyruvic acid interacts on the oxidative decarboxylation of tne latter to homogentisic acid.

SUMMARY

1. The enzyme system concerning the oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid has been obtained from the rabbit liver in a soluble and purified state.

2. The optimum pH for the enzyme activity is pH 7.4; the activity of the 30 to 50 per cent fraction fractionated with ammonium sulfate is the highest.

3. The enzymic oxidation of *p*-hydroxyphenylpyruvic acid is inhibited completely by oxin, NaN_3 and H_2S; partially by monoiodoacetic acid; but not by KCN, *a*, *a*′-dipyridyl or CO. The enzyme preliminarily inhibited by oxin regains its activity by the addition of V. B_{12}, V. C and methylene blue; but not by Fe^{++} and folic acid.

4. The resolved purified enzyme regains its activity when supplemented with both V. B_{12} and V. C; whereas folic acid and bivalent metal ions have no effect. A definite concentration of V. B_{12} for achieving a maximum rate of restoring the enzyme activity has been determined.

5. 2,5-Dihydroxyphenylpyruvic acid has been proven to be the metabolite of *p*-hydroxyphenylpyruvic acid through investigation by paperchromatography of the reaction mixtures that involve purified enzymes restored by V. B_{12} plus V. C.

6. It has been noticed that, in the presence of V. C, V. B_{12} serves as the coenzyme for the enzyme oxidizing *p*-hydroxyphenylpyruvic acid to 2,5-dihydroxyphenylpyruvic acid and suggested that the enzyme is an oxidase.

7. 2,5-Dihydroxyphenylpyruvic acid is converted to homogentisic acid through oxidative decarboxylation by the resolved purified enzyme supplemented with DPN and co-carboxylase.

8. The oxidation of *p*-hydroxyphenylpyruvic acid interacts on the oxidative decarboxylation of 2,5-dihydroxyphenylpyruvic acid.

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STUDIES ON GLUCOSE DEHYDROGENASE. II

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Harrison (*1*) discovered in the mammalian liver an enzyme, glucose dehydrogenase. Mann (*2*) showed that this enzyme required a co-enzyme for its activity. On the specificity of glucose dehydrogenase towards co-enzymes, many workers (*3, 4, 5, 6, 7*) perceived an interesting phenomenon that both DPN and TPN act as hydrogen acceptors. Purification of the enzyme from acetonized liver powder was performed first by Harrison (*1*) and later by Strecker and Korkes (*7*). Wainio and Brunelli (*8, 9*) purified the enzyme from water extracts of lyophilized lamb liver. The problem of the substrate specificity of the enzyme has not been entirely solved in detail by the several works hitherto undertaken (*7, 8, 10, 11*). Eichel and Wainio (*12*) found that glucose dehydrogenase was activated slightly by addition of catalase and sodium malonate, and was inhibited by ethyl urethane and sodium iodoacetate. But Harrison (*1*) did not recognize any inhibition of this enzyme by iodoacetate. Euler and Adler (*13*) reported that $7.6\times10^{-5}M$ Cu^{++} inhibited this enzyme by about 50 per cent and Müller (*14*) reported this enzyme to be insensitive to cyanide.

Ochoa *et al.* (*15–20*) reported a synthesis of malate from pyruvate and carbon dioxide by coupling with the malic enzyme with glucose-6-phosphate dehydrogenase system. In this paper the author reports on the purification of glucose dehydrogenase from fresh bovine liver. Data are presented on the substrate specificity of the purified enzyme, the inhibitory or activatory effects of several chemicals on it and other properties of it. And then it was attempted to obtain a relatively large amount of oxalacetate from pyruvate and CO_2 by coupling the glucose dehydrogenase system as an energy donating system with oxalacetic decarboxylase system. The data obtained appear to indicate that the glucose dehydrogenase system does not couple with the oxalacetic decarboxylase system, but that oxalacetate is formed by the co-existence of malate to some extent.

EXPERIMENTAL

Optical Assay and Enzyme Unit—The activity of glucose dehydrogenase was quantitatively measured by the colorimetric method reported formerly by the author (*21*). One enzyme unit is defined as an amount of enzyme sufficient to cause an increase in optical density at 495 mμ of 0.1 per mg. of nitrogen per 15 minutes (0.5 cm. light path). The total nitrogen content of the enzyme solution was determined by Kjeldahl's method.

Purification of Enzyme

All the following procedures were carried out at approximately 0–10°.

Extraction—A 2000 g. portion of fresh bovine liver was minced and homogenized with 2000 ml. of ice-cold deionized water, and vigorously shaken for 45–60 minutes by constant stirring. The suspension obtained was adjusted to pH 5.4–5.6 by addition of *N* acetic acid and, after standing for 30 minutes, centrifuged (3000 r.p.m., 40 minutes). The supernatant solution was filtered through four layers of cheesecloth in order to remove the fat membrane separated on the surface. The precipitate was again extracted with 1000 ml. of distilled water.

First Fractionation by Ammonium Sulfate—To the combined extract (specific activity*: 0.6), ammonium sulfate was added slowly by constant stirring (17.7 g./100 ml.). After standing for 15 minutes in the cold, this solution was centrifuged (3000 r.p.m., 40 minutes). The supernatant solution (2850 ml.) was adjusted to pH 5.4 with *N* acetic acid, ammonium sulfate was added slowly (21 g./100 ml.) (65 per cent saturation), and after standing for 2 hours or sometimes overnight, the mixture was centrifuged. The precipitate was dissolved in about 400 ml. of 0.05 *M* phosphate buffer (pH 7.4). The solution was dialyzed for 60 hours against running tap water and then further dialyzed for 12 hours against distilled water. Any precipitate which formed during dialysis was discarded. (Over-all yield: 95–90 per cent; specific activity: 2.4).

Heat Trearment—The dialyzed solution (about 3100 ml.) was placed in a water bath at 46–47° for 5 minutes. Then the mixtures was rapidly cooled 10° and centrifuged (3000 r.p.m., 30 minutes). The precipitate was discarded. (Over-all yield: 75–80 per cent; specific activity: 3.5).

Acid Treatment—The above solution (about 3000 ml.) was adjusted to pH 5.0 with *N* acetic acid. After standing for 1 hour, the solution was centrifuged (3000 r.p.m., 30 minutes). Some activities coprecipitated with non-enzymatic protein were recovered by washing the precipitates with a small amount of 0.1 *M* phosphate buffer (pH 7.4). (Over-all yield: 60–70 per cent; specific activity: 4.2).

Second Fractionation by Ammonium Sulfate—The combined solution (2900 ml.) was adjusted to pH 5.6 with *N* acetic acid, and ammonium sulfate was added slowly (26.9

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* Since the combined extract at this stage containes a large amount of hydrogen donators, the extract was further dialyzed against running tap water and distilled water for 72 hours at 0° and the specific activity was measured without discarding the precipitate formed during dialysis.

g./100 ml.) under constant stirring. After standing for 1 hour, the mixture was centrifuged (3000 r.p.m., 40 minutes). To the supernatant solution, ammonium sulfate was added (12.8 g./100 ml.). After standing for 1 hour, the mixture was centrifuged (3000 r.p.m., 40 minutes). The precipitate was dissolved in 450 ml. of distilled water and dialyzed overnight against distilled water. Any precipitate formed was discarded. (Over-all yield: 58 per cent; specific activity: 8.7).

*First Treatment by Calcium Phosphate Gel**—The enzyme solution (500 ml.) was diluted with an equal volume of distilled water and adjusted to pH 5.8 with 10 per cent acetic acid. A half volume** of calcium phosphate gel (45.0 mg./ml., dry weight) was added to the enzyme solution. After continuous stirring for 30 minutes, the suspension was centrifuged and the gel discarded. (Over-all yield: 50 per cent; specific activity :10.2).

Second Treatment by Calcium Phosphate Gel—The enzyme solution (980 ml.) was adjusted to pH 5.4 with 20 per cent acetic acid. The following procedure was employed for complete adsorption of enzyme upon calcium phosphate gel: A portion of 1000 ml. of calcium phosphate gel was centrifuged. The sedimented gel and 980 ml. of the enzyme solution were mixed together and homogenized. Then the mixture was stirred continuously for 30 minutes and centrifuged (3000 r.p.m., 40 minutes). The precipitate was suspended in 250 ml. of 0.2 M phosphate buffer (pH 7.4) and stirred vigorously for 30 minutes. The mixture was centrifuged and the residue was re-eluted with 150 ml. of 0.2 M phosphate buffer (pH 7.4) as before. (Over-all yield: 33 per cent; specific activity: 15.8).

The solution thus obtained was used in the experiments reported here except when otherwise stated. This enzyme solution could be stored in a frozen state without much loss of activity for about three months in an ice-box. When a more concentrated enzyme solution was needed, the enzyme solution was adjusted to pH 5.6, and ethanol was added at −5° to reach a final concentration of about 30 per cent, and after standing for 15 minutes in the cold, the mixture was centrifuged (3000 r.p.m., 20 minutes). The precipitate was dissolved in 0.05 M phosphate buffer (pH 7.4) in a desired concentration, or was dried on a porous tile and stored in a desiccator. The experimental results of purification are summarized in Table I.

Properties of Enzyme

As shown in Table I, the preparation of glucose dehydrogenase could not be much raised in purity by the method described above. The absorption spectrum of the purified glucose dehydrogenase solution was obtained by Beckman spectrophotometer as illustrated in Fig. 1. From the maximum light absorption at 406 mμ, it was assumed that this enzyme solution might contain some heme proteins. The catalase activity of this enzyme solution was measured by the rapid-drop method contributed by Shirakawa (*23*). Catalase activity was expressed with monomolecular velocity con-

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* Calcium phosphate gel was prepared by Utkin's method (*22*).

** The exact amount of the calcium phosphate gel required for the present purpose was varied from 0.3 to 0.5 volumes.

TABLE I

Purification of Glucose from Fresh Bovine Liver

	Volume of solution	Total enzyme unit	Nitrogen content	Specific activity	Yield
	ml.	*unit*	*mg.*	*units per mg. nitrogen*	*per cent*
Water extracts	3800	19,250	27,500	0.6	100
First ammonium sulfate fractionation	3100	18,800	7,834.4	2.4	95
Heat treatment	3000	17,500	5,004	3.5	80
Acid-treatment	2950	17,400	4,147	4.2	65
Second ammonium sulfate fractionation	500	9,848	1,132.4	8.7	53
First calcium phosphate adsorption	980	9,500	953.6	10.2	50
Second calcium phosphate treatment	430	6,478	410.0	15.8	34

stant, "k", and k-value of this enzyme solution was 0.00039.

$$k = \frac{1}{t(\mathrm{sec})} \log_{10} \frac{a}{(a-x)}$$

Substrate Specificity—With the purified preparation, the following substances were found to be inactive as substrates: lactose, maltose, glucose-1-phosphate*, glucose-6-phosphate*, D-fructose, sucrose, quercitol, D-ribose, inositol. But-D-glucose, D-xylose, L-xylose and Na gluconate reduced TTB under the conditions previously reported, indicating that these substances may be oxidizable. The reduction rate of TTB for xylose was about one fourth of that for glucose. Galactose and arabinose reduced TTB but slightly. (Table II).

Factors which Affect Enzyme Activity—The influences of substrate concentration and of enzyme concentration on the reaction rate were formerly reported (*21*). The reaction velocity is directly proportional to the enzyme concentration under the certain conditions described previously.

(i) Effect of temperature on enzyme stability: Each 1 ml. aliquot of aqueous enzyme solution was placed in Thunberg tubes which placed in the water bath at certain temperatures for 5 minutes, and then rapidly cooled down to 10°. Then other components, such as DPN, flavoprotein, phosphate buffer, substrate, TTB and, if necessary, some other chemicals were added and the enzyme activities were measured at 37°, by the ordinary method. It was found that the destruction temperature of this enzyme was about 50°.

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* The author is indebted to Mr. Sonoda for his preparation of glucose-1-phosphate and glucose-6-phosphate.

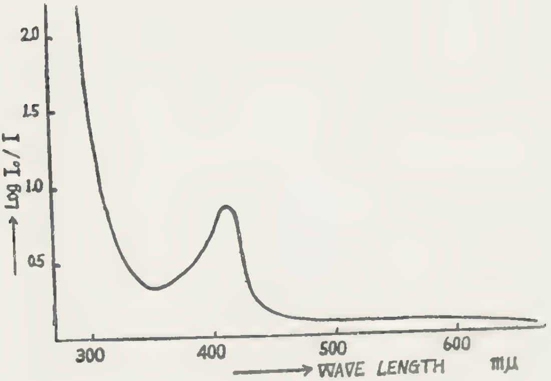

FIG. 1. Absorption curve of purified glucose dehydrogenase preparation.

TABLE II

Specificity of Glucose Dehydrogenase to Sugars

Substrates	Concentration	Optical density
	M	
D-Glucose	0.2	1.52
D-Galactose	0.2	0.25
D-Mannose	0.2	0.13
D-Xylose	0.2	0.40
L-Xylose	0.2	0.35
D-Arabinose	0.2	0.25
D-Fructose	0.2	0.10
Quercitol	0.2	0.09
Inositol	0.2	0.10
D-Rhamnose	0.2	0.08
Lactose	0.2	0.14
Glucose-1-phosphate	0.2	0.12
Glucose-6-phosphate	0.2	0.16
Na gluconate	0.125	0.33

Total volume, 5.0 ml.: substrate, 0.5 ml.; glucose dehydrogenase solution, 1.0 ml. (specific activity 15.8); coenzyme, 0.5 ml. of 1 per cent solution of DPN in water; flavoprotein (Straub), 0.5 ml.; buffer, 1.0 ml. of 0.2 *M* phosphate at pH 7.4; oxidation-reduction dye, 0.5 ml. of 0.5 per cent solution of TTB in water; and distilled water. Temperature: 37°.

(ii) The optimum pH of glucose dehydrogenase and its determination: In these experiments the buffer solution containing 0.2 *M* Na_2HPO_4 and 0.2 *M* KH_2PO_4 was

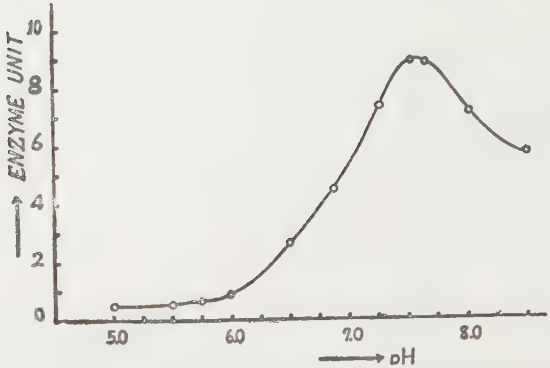

FIG. 2. pH-Activity curve of glucose dehydrogenase system. Reaction mixtures: 1.0 ml. of buffer as described in the text, 1.0 ml. of glucose dehydrogenase (specific activity: 9.8), 0.5 ml. of 1 per cent DPN, 0.5 ml. of flavoprotein, 0.5 ml. of 2 *M* D-glucose and 0.5 ml. of 0.5 per cent aqueous solution of TTB.

TABLE III

Effects of Various Substrates on the Glucose Dehydrogenase Activity

Addition	Concentration	Optical density	Effect in percentage
	M		%
None*		1.25	
D-Galactose	0.2	1.30	+ 4
D-Arabinose	0.2	1.22	+ 2
D-Mannose	0.2	1.24	+ 0.8
D-Rhamnose	0.2	1.25	0.0
D-Xylose	0.2	1.40	+12
L-Xylose	0.2	1.42	+12
Mannit	0.2	1.29	+ 3
Quercitol	0.2	1.27	+ 1
Maltose	0.2	1.20	− 4
Sucrose	0.2	1.18	− 6
Lactose	0.2	1.28	+ 2
Raffinose	0.2	1.19	− 5

* Complete system without addition was made using: 1.0 ml. of glucose dehydrogenase (specific activity, 12.9), 0.5 ml. of 1 per cent DPN, 0.5 ml. of Straub's flavoprotein, 1.0 ml. of 0.2 *M* phosphate buffer (pH 7.4) in the main tube of a Thunberg tube; 0.5 ml. of 2 *M* D-glucose, 0.5 ml. of 0.5 per cent TTB in the side arm. Condition: anaerobic; temperature at 37°; reaction time for 15 minutes.

used. The pH-values were measured with a colorimeter. The optimum pH of this enzyme was found at about 7.4–7.6. (Fig. 2).

TABLE IV

Effects of Some Inorganic and Organic Compounds on Glucose Dehydrogenase Activity

Inhibitors and activators added	Final concentration	Per cent of activation (+) and inhibition (−)
	M	%
$CoCl_2$	1×10^{-3}	+ 6
$MgCl_2$	1×10^{-3}	− 5
$ZnCl_2$	1×10^{-3}	− 5
$AgNo_3$	1×10^{-3}	−100
$CuCl_2$	1×10^{-4}	−100
$FeCl_2$	1×10^{-3}	− 30
$SnCl_4$	1×10^{-3}	− 4
$AlCl_3$	1×10^{-3}	− 6
$Pb(COO)_2$	1×10^{-3}	− 8
o-Quinone	9.3×10^{-3}	−100
p-Quinone	9.3×10^{-3}	−100
Maleic acid	1×10^{-2}	− 10
Naiodoacetate	1.5×10^{-2}	− 5
AsO_3	1×10^{-2}	− 7
Oxidized glutathione	2×10^{-3}	0
Cysteine	1×10^{-3}	0
Nitrogen mustard	1×10^{-2}	−100
Urethane	3×10^{-1}	− 22
NaF	1×10^{-2}	0
Tea-leaves extract	(20 mg.)	−100
D-Ascorbic acid	5.7×10^{-3}	− 20
Pyrocatechol	1×10^{-2}	−100
KCN	1×10^{-2}	0
Na gluconate	1×10^{-1}	+ 15
Na malonate	1×10^{-1}	+ 2

Complete system is made as shown in Table I.

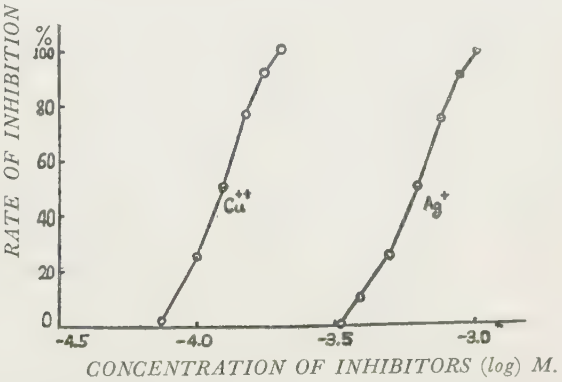

FIG. 3. Inhibition by Cu^{++} and Ag^{+}. For details see text.

Inhibition of Glucose Dehydrogenase—There was observed no competitive inhibitory effect on glucose dehydrogenase, in the presence of D-galactose, D-arabinose, D-mannose, D-xylose, L-xylose, mannit, quercitol, D-fructose, maltose, sucrose, lactose, or raffinose, (Table III). It was found that glucose dehydrogenase was inhibited by copper and silver ions, and slightly so by ferrous ions, (Table IV and Fig. 3). As shown in Table IV, most SH-reagents did not show any recognizable inhibitory action, but what was very interesting was that the quinonoids exhibited certain inhibitory effects. Fresh homogenized tea-leaves pyrocatechol, urethane and nitrogen mustard inhibited the glucose dehydrogenase activity. It is well known that a highly concentrated reaction product of substrate inhibits the reaction velocity of dehydrogenase. But in this case, sodium gluconate did not inhibit the activity of this enzyme. This may have been due to the existence of some impure enzyme which oxidize gluconate, as described above (Table II).

Chromatographic Identification of the Oxidation Product of Glucose by Glucose Dehydrogenase—Harrison (*24*) isolated the oxidation product of glucose by glucose dehydrogenase. The present author attempted chromatographically to prove the oxidation product to be gluconic acid.

Filter paper used: Toyo Roshi, No. 2.

Developing solvents used: Solvent mixture A, *n*-butanol-glacial acetic acid-water (4 : 1 : 2, volume ratio); Solvent mixture B, *n*-butanol-formic acid-water (4 : 1 : 2, volume ratio).

Spraying reagent used: 0.04 per cent (*w/v*) aqueous solution of bromthymol-blue adjusted to pH 7.4 with NaOH (*25*).

Preparation of gluconic acid: Calcium gluconate was synthesized by the method of Kiliani (*26*) and recrystallized from water. Calcium gluconate was identified as a gluconic acid benzimidazole by the method of Moore and Link (*27*). An aqueous solution of calcium gluconate was poured into a columna of cation exchange resin and a highly acidic effluent was obtained (pH 1.3). This effluent was left standing overnight in an ice-chest and then concentrated to about 1.0 *M*.

Preparation of oxidation product: The oxidation of glucose by glucose dehydrogenase was carried out as follows: 20 ml. of a purified glucose dehydrogenase solution (specific activity: 15.8), 5 ml. of 1 per cent DPN, 5 ml. of Straub's heart flavoprotein, 10 ml. of 2 *M* of D-glucose, and 5 ml. of 0.5 per cent TTB aqueous solution were mixed up in a flask, and this flask was set in a water-bath (37°). The reaction was allowed to proceed for 3 hours. The reaction mixture was then boiled for 10 minutes and the proteinous precipitates were filtered off. The cation exchange resin was added to the filtrate. The acidic solution was left standing overnight in an ice-box. Next day, the solution was concentrated and the residue was used as a reaction product.

The usual method was employed in ascending paper strip chromatography. The difference between solvent mixtures A and B in the extent of formation of "tales" and "beards" on the gluconic acid spot was studied, and solvent mixture B was found to better for the present purpose. Comparison of experimental strips with paper strips containing gluconic acid, glucose plus gluconic acid, glucose, and H_3PO_4 demonstrated the formation of gluconic acid in the enzymatic dehydrogenation of

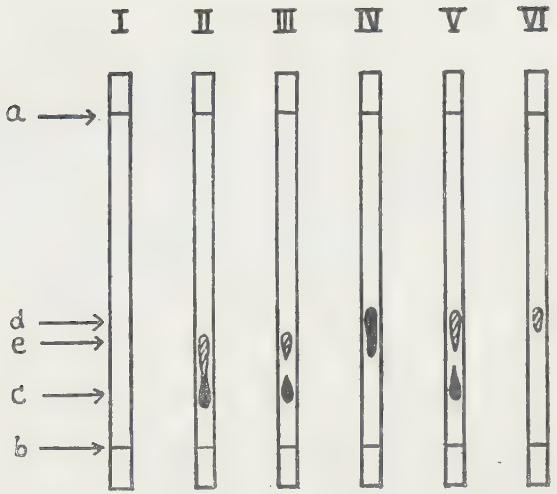

FIG. 4. Paper chromatographic identification of gluconic acid. (a) solvent front, (b) start of chromatograph, (c) gluconic acid zone, (d) H_3PO_4 zone, (e) zone obscure; I, D-glucose; II, gluconic acid (1 *M*); III, glucose plus gluconic acid; IV, H_3PO_4; V, oxidation product with enzyme and D-glucose; VI, reaction prodcct with enzyme and water instead of D-glucose. After allowing the ascending chromatgram to develop for 24 hours in sealed cylinder at 25°, the strips are dried in a stream of warm air and sprayed with BTB solution.

glucose (Fig. 4). The Rf values of standard gluconic acid and oxidation products were 0.148 and 0.149, respectively, when solvent mixture B was used. On the other hand, no attempt to identify the reaction product in the form of its benzimidazole compound has so far succeeded.

A Question whether the Glucoso Dehydrogenase System Does Couple with the Oxalacetic Decarboxylase System or not

Preparation of Oxalacetic Decardoxylase—Micrococcus lysodeikticus (*28, 29*) was selected as the enzyme source of oxalacetic decarboxylase, because the preparation of oxalacetic decarboxylase from the *M. lysodeikticus* is free from malic enzyme and oxalsuccinic decarboxylase (*30, 31, 32*). *Lactobacillus arabinosus* (strain 17–5) was inadequate for the purpose, because the enzyme preparation from this microorganism is analogous to malic enzyme in action (*33*).

*M. lysodeikticus** was cultured in the following medium: agar 2 per cent, peptone 1 per cent, beef extract 1 per cent, NaCl 0.1 per cent, tap water 10 per cent, plus a sufficient amount of distilled water. A small amount of *n*-octyl alcohol was added to prevent foaming on aeration. The medium was autoclaved for desinfection. Plate agars were incubated for 72 hours at 25°. The yield of wet cells amounted to about 10 g. per liter of the medium. The cells were washed with physiological saline solution, and lysed by incubation with egg white (2 g. per 1 g. wet bacteria) at 37°. The lysis was readily completed after 60 minutes incubation as observed microscopically. This lysed preparation was adjusted to pH 5.0 by addition of *N* acetic acid and the precipitate was discarded after centrifugation. To 120 ml. of the supernatant solution, about 10 ml. of aluminium hydroxide (*34*) were added, and the mixture was continuously stirred for 30 minutes. This mixture was then centrifuged and the precipitate discarded. To one volume of the supernatant solution were slowly added 3 volumes of acetone at 0°, and the mixture was centrifuged. The precipitate was dried in a desiccator over H_2SO_4. This acetone-dried powder was used as oxalacetic decarboxylase preparation in the present experiments.

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* For the supply of the stump used, thanks are due to the Laboratory of Bacteriology, Faculty of Medicine, Nagasaki University, Nagasaki, Japan.

Methods. The fixation of CO_2 was measured by manometric technique (*35*) and the determination of oxalacetate by the aniline-citrate method of Edson (*36*).

The oxalacetic decarboxylase prepared as mentioned above was active, CO_2 evolution being 690 *μl.* per 30 minutes per 50 mg. of enzyme preparation. The results given in Table V appear to indicate that addition of malate to glucose dehydrogenase system promotes the formation of oxalacetate, but the glucose dehydrogenase system without co-presence of malate does not promote it. The author attempted to identify oxalacetate in the reaction mixture by chromatographic technique (*31*), but without success.

TABLE V

Effects of the Addition of Glucose Dehydrogenase System to the Oxalacetate Carboxylation Reaction

Reaction system	Oxalacetate formed
	μl.
Complete system*	92
No malate	37
No glucose	28
No DPN	29
No $MgSO_4$	48
No glucose dehydrogenase solution	32

* 0.5 ml. of 0.2 *M* phosphate buffer (pH 7.4), 0.5 ml. of $1.65\times10^{-1}M$ bicarbonate, 0.5 ml. of 1.5 *M* pyruvate, 0.5 ml. of 1 per cent DPN, 0.5 ml. of $5\times10^{-2}M$ $MgSO_4$, 0.5 ml. of 2 *M* D-glucose, 1.0 ml. of purified glucose dehydrogenase (specific activity; 15.0), 0.5 ml. of 0.5 *M* malate, and 50 mg. of oxalacetate decarboxylase preparation; total volume, 5.0 ml.; 5 per cent CO_2 in N_2 in gas space; temperature at 30°.

DISCUSSION

To study the role of sulfhydryl groups in glucose dehydrogenase the author used the following sulfhydryl reagents: oxidized glutathione, sodium iodoacetate, maleic acid, *o*-quinone, *p*-quinone and AsO_3. It would be impossible to determine only from the results whether this enzyme protein has any active SH-groups or not. Needless to add, the inhibition by quinonoids was also demonstrated by the classical Thunberg technique (*35*), but no activation by cyteine was ever observed. Therefore, the author could not verify Eichel and Wainio's results. The failure of inhibition of this enzyme by sodium malonate proved that the succinic-fumaric system was not involved in the dehydrogenation by glucose dehydrogenase system.

Little evidence has so far been obtained for final settlement of the physiological significance of glucose dehydrogenase in mammalian tissues. It was suggested that the glucose-6-phosphate and 6-phosphogluconic systems in photo-chemosynthesizing organisms act significantly as an alternative pathway in the synthesis of glucose from pentose (*7*, *37*). The idea that the glucose dehydrogenase system may also play a similar role in the synthesis of glucose from pentose, tetrose or triose, is attracting attention because of the fact that the following reaction catalyzed by glucose dehydrogenase is reversal in the presence of glucose dehydrogenase (*7*).

$$\text{Glucose}+\text{DPN}_{\text{oxid.}}\rightleftharpoons\text{glucose}+\text{DPN}_{\text{red.}} \quad \text{............(1).}$$

The oxidation reduction potential of reaction (1) was reported to be -0.44 volt, from which the free energy (ΔF) was found to be -7114 cal. per mole (*7*). This value of ΔF is nearly in the same order of magnitude as that of glucose-6-phosphate dehydrogenase system (-6890 cal. per mole). The fact that an intimate resemblance in ΔF values has been seen between these two systems has attracted the author's attention:

Wood and Werkman (*38*, *39*), in the course of their investigations of the CO_2 utilization by propionic bacteria, found the following reaction:

$$\text{Oxalacetate}\rightleftharpoons\text{pyruvate}+CO_2 \quad \text{..................................(2).}$$

Since the equilibrium of this reaction (2) is so far in the direction of decarboxylation, it is improbable that a large amount of oxalacetate is formed by carboxylation of pyruvate as a primary reactant. Therefore, it is highly probable that the coupling of the reaction with an energy yielding reaction may shift the reaction (2) to the left. It was confirmed by the isotopic procedure that the reaction (2) is fairly reversible (*40*, *41*, *42*), but the interrelation between the malic enzyme and the oxalacetic decarboxylase is unknown (17, 18, 31, 40–47). The coupling of the glucose dehydrogenase system with the oxalacetic decarboxylase system was not confirmed in the present experiments, but the formation of oxalacetate was observed to some extent in the presence of the mixed substrate (malate and pyruvate) under the conditions described above and shown in Table V. The reaction mechanism for oxalacetate formation remains as yet obscure, and further works on the purification of enzymes are required to solve the mechanism of this reaction. It appears that no free energy might be transferred from the glucose dehydrogenase system to the oxalacetate decarboxylase system and that there would be required some other suitable energy transferring system, such a system as suggested by Kalnitsky and Werkman (*20*), or a dismutation system, such as TPN-linked dismutation in the presence of malic enzyme.

SUMMARY

1. The purification of glucose dehydrogenase from fresh bovine liver has been described. D-Xylose, L-xylose, and Na gluconate were oxidized at a slower rate than D-glucose by this purified preparation.

2. It was found that glucose dehydrogenase is inhibited by quinonoids, nitrogen mustard, Cu^{++}, Ag^{+}, urethan, catechin and tea-leaves extract, but not by NaF, D-ascorbic acid, cyanide, other metal ions than Cu^{++} and Ag^{+} ions, several sugars such as fructose, galactose, D-xylose, L-xylose, arabinose, quercitol, sucrose, and mannitol, nor by any such SH-reagents as malic acid, AsO_3, oxidized glutathione, sodium iodoacetate, or by several other reagents.

3. It was ascertained by paperchromatographic technique that the primary oxidation product of glucose by liver glucose dehydrogenase is gluconic acid.

4. Although it appears that the glucose dehydrogenase system does not link directly with oxalacetic decarboxylase system, a small amount of oxalacetate was detected only in the co-existence of malate. But the mechanism of this phenomenon is unknown.

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DIFFERENTIAL STAINING OF NUCLEIC ACIDS

IV. QUANTITATIVE STUDY ON THE METACHROMASIA OF THIONIN CAUSED BY NUCLEIN ACIDS[1]

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(Received for publication, October 2, 1953)

In a previous paper of this series (*1*), it was pointed out that nucleic acids of whatever type gave rise to metachromasia of thionin on entering electrostatic combination with this dye. The metachromasia shows in two directions: higher molar ratios of nucleic acid P to thionin result in shifting of the absorption maximum towards the longer wavelengths (α-color), while lower ones cause shifting towards shorter wavelengths (β-color). These two colorations are observed in tissue sections stained with thionin: whereas PNA stains in β-color, DNA tends to stain in α-color, and the latter staining has been ascribed to the inhibition of staining reaction caused by competitive action of basic proteins associated with DNA.

An interesting example of metachromatic differentiation of nucleic acids in tissue staining was reported by Flax and Himes (*2*): azure phthalate stains PNA red and DNA blue. These authors considered that the mechanism of differentiation resides in the difference in molecular structure of DNA and PNA. In order to test whether any difference exists between different types of nucleic acids with respect to the intensity of metachromasia, a quantitative spectrophotometric study on the metachromasia of thionin has been undertaken with purified samples of nicleic acids.

MATERIALS AND METHODS

The sample of PNA (Na salt) used was the same as in the previous papers (*1*, *3*). A purified sample of "non-disaggregated" or "highly associated" DNA (pDNA)

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[1] Aided in part by the Grants for Scientific Research of the Ministry of Education given to the Synthetic Research Group on "Nucleic Acids."

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(Na salt) obtained from calf thymus was the gift of Dr. T. Miyaji, the 1st Department of Pathology, Osaka University Medical School. Used as the "disaggregated" DNA (dDNA) was a purified, but powderly sample of herring sperm DNA (Na salt), which was kindly supplied by Dr. I. Watanabe, Institute for Science and Technology, University of Tokyo. This was extracted by the Pollister and Mirsky (*4*) method but, probably owing to the use of non-fresh material, could not be obtained in fibrous form. The same batch of recrystalized thionin as used before (*1*, *5*) was employed. Determination of phosphorus content of nucleic acid solutions was carried out by the method of Gomori (*6*, *7*).

A 2 ml. portion of $5\times10^{-5}\,M$ solution of thionin (13.10 mg. of thionin per liter) was pipetted into a series of test tubes and mixed with varying volumes of respective nucleic acid solutions of appropriate concentrations so as to change the molar ratio of nucleic acid P to thionin (R) 20 through 0.25. The volume of the mixture in each tube was adjusted to 3.6 ml. by adding distilled water. The concentration of thionin in all tubes was thus fixed at a constant level of $2.78\times10^{-5}\,M$. A simple thionin solution of the same concentration without nucleic acid added served as the control. The mixtures with different R values gave the coloration ranging from blue to purple red, and with the decrease of R below unity, the mixture progressivelr lost the reddish tinge to approximate the violet color of the control. The spectrophotometric determination was made with Beckman spectrophotometer Model DU.

RESULTS

As shown in a previous paper (*5*), high R values give α-color of thionin, the absorption peak being relatively sharp and situated at 610–620 mμ. With the decrease of R, the peak tends to flatten and the maxmium shifts towards shorter wavelengths; it is thus located at 570 mμ in typically β-colored mixtures. Control tube with thionin alone gives a sharp peak at 600 mμ.

In Fig. 1 are plotted molecular exitnction coefficients of thionin (ε) at 610 and 570 mμ in various nucleic acid-thionin mixtures against R. It is seen this figure that the curve of ε_{610} takes a V-shape for different nucleic acids, the minimum value always being at $R=1$. The right arm of the curve represents the gradual increase of bluish tone or α-color metachromasia with the increase of R. Starting from pure thionin solution ($R=0$), the grandual increase of R would result in the increase in number of thionin molecules which are bound to nucleic acid saturating all the ayailable sites (phosphoric acid residues). Such a state of thionin molecules should give rise to the metachromasia to β-color and greatly reduce the value of ε. Thus, the rise of the left arm of the ε_{610} curve means the increase of free thionin molecules remaining uncombined with nucleic acid. The point where ε takes the minimum value should then indicate the quantity of nucleic acid P just sufficient to bind all the thionin molecules. Accordingly, R which gives minimum ε represents the stoichiometry of the reaction between thionin and nucleic acid. Since this R value is around unity for PNA, pDNA and dDNA, the compound of the reaction consists of one mole dye per each nucleic acid P with every type of nucleic acids.

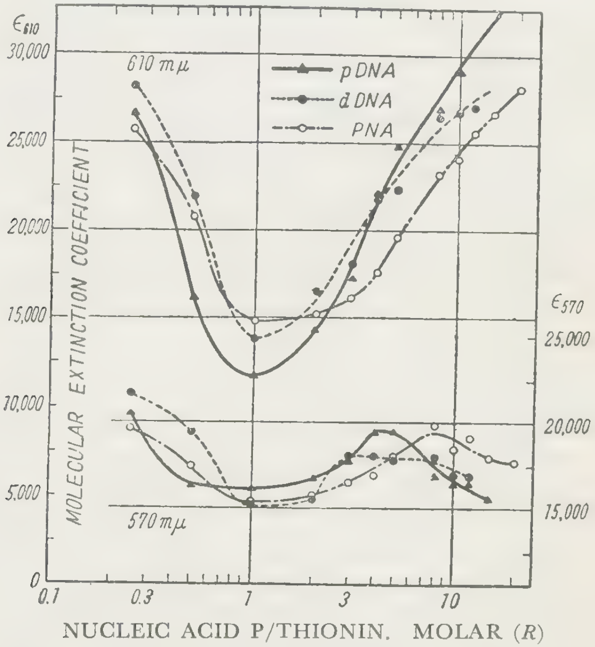

FIG. 1. Change in molecular extinction coefficient of thionin at various values of molar ratio of nucleic acid P to the dye. Determined at the level of $2.78 \times 10^{-5} M$ of thionin.

The corresponding curves of ε_{570} also show the minimum value at $R=1$. But, in this case, the slope of the curves is less steep, and takes rather a complicated shape at higher R values. It can be said that the difference of α- and β-colors of the mixtures consists mainly in the change in ε_{610} rather than ε_{570}.

Visual inspection of the series of tubes from extreme α- ($R>20$) to typical β-color ($R=1$) gave an impression that the bluishness of pDNA mixtures at higher R values is more intensive than that of PNA mixtures, but, at the same time, the reddishness of pDNA mixtures at lower R values is likewise stronger than the corresponding one of PNA. And at $R=3$, the PNA mixture is definitely violet whereas pDNA mixture is blue. It should thus be concluded that there is difference to a certain extent in the metachromasia-inducing ability between pDNA and PNA. Whether this difference is caused by the difference in molecular structure (arrangement of mononucleotides) or rather by the "molecular" weight of nucleic acids could be answered by experiment with dDNA.

To express the intensity of metachromasia of DNA's comparative with that of PNA, we may use the "increment" of metachromasia (I) at a given R value as defined by the formula:

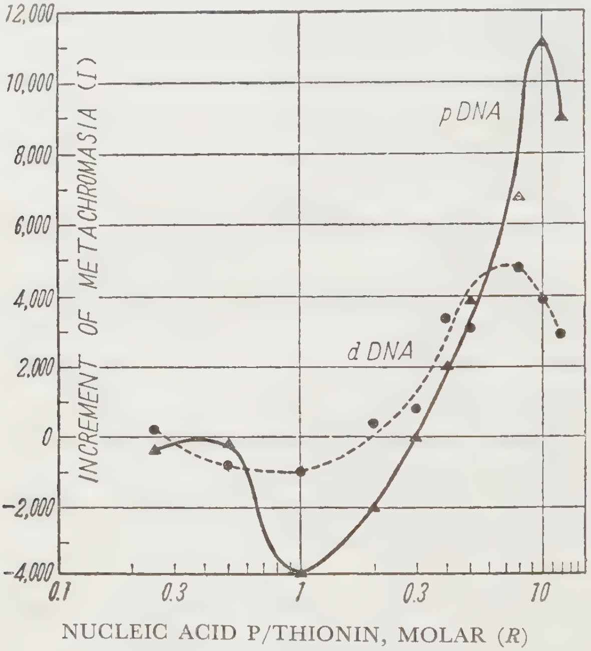

FIG. 2. "Increment of metachromasia" of thionin (I), or comparison of the intensities of metachromasia caused by non-disaggregated and disaggregated DNA's which are measured by the differences of PNA and respective DNA's in molecular extinction coefficients at 610 and 570 mμ. Calculated from the data given in Fig. 1 by the formula given in the text.

$$I=(\varepsilon_{610}\mathrm{DNA}-\varepsilon_{610}\mathrm{PNA})-(\varepsilon_{570}\mathrm{DNA}-\varepsilon_{570}\mathrm{PNA}).$$

The first term in parentheses of this formula means the bluishness and the second term the reddishness of thionin-DNA complex in excess of those of thionin-PNA complex. In Fig. 2, I is plotted against R for pDNA and dDNA. The "molecular" weight of dDNA used here is unknown, but it forms a pink precipiteate from aqueous methyl green-pyronin (*cf.*, *3*, *5*). The positive or negative I values indicate that the bluishness (reddishness) of thionin color is enhanced (reduced) or reduced (enhanced), respectively, more strongly by DNA than by PNA. Fig. 2 clearly shows that DNA is more metachromatic than PNA in either direction. That is, DNA augments the metachromasia into α-color at higher R values, and the one into β-color at lower R values more extensively than PNA. However, this trend is much more conspicuous with pDNA than dDNA. This means that the difference in the intensity of metachromasia between DNA and PNA depends, at least in part, upon the "molecular" weight of the nucleic acids.

DISCUSSION

The quantitative study on the metachromasia of thionin induced by nucleic acid as presented in this paper reveals that the intensity of metachromasia is stronger with pDNA than with PNA. A part at least of this difference consists in the difference of "molecular" weight of these two nucleic acids. Difference in the intensity of metachromasia between pyroninophilic dDNA and PNA is less conspicuous. The dDNA to be obtained through drastic tratment might not show any significant change from PNA in the intensity of metachromasia. Whether there is some ultimate quantitative difference of metachromasia between PNA and DNA, which is to be due to the difference in molecular structure, can not be answered in the scope of the present experimentation.

In a previous paper of this series, the α-color of chromatin in thionin-stained tissues was interpreted as an expression of the competitive interference by nuclear proteins of the staining reaction, which results in the reduction in number of bound thionin molecules per DNA P, leading to the appearance of α-color of thionin. However, since it was revealed that at certain R values the color of thionin bound to DNA is potentially more bluish than that with PNA, it could not be excluded that the α-color of chromatin is at least partially due to the highly associated DNA *per se*. This is especially true in cases where the color difference between PNA and DNA in the same cells is not so conspicuous.

Indeed, the thionin color taken by cellular PNA is variable. In certain species of insects (Orthoptera), the β-color of cytoplasmic and nucleolar PNA is strongly reddish, in parencyhmal cells of rat liver it is less, but yet distinctly, reddish, and in plasma cells or reticulum cells of rat spleen it is rather violet. From these findings it would be deduced that also the staining reaction of PNA with thionin may undergo the interference, probably due to proteins, to varying extents in different cells or eventually in cells of different physiological activities. And in the case where PNA stains violet and DNA blue, like in some basophilic cells in spleen, there is a possibility that interference of staining reaction takes place even to comparable extents with both types of nucleic acids. Therefore, in such cases the mechanism of differential staining of nucleic acids may consists in the potentially different metachromatic abilities of different nucleic acids rather than the differential effects of proteins bound to respective types of nucleic acids.

It can now well be anticipated that the same or similar situation hold with the metachromatic differentiation of nucleic acids with azure phthalate of Flax and Himes (*2*).

Though a potential difference in the metachromatic nature of two types of nucleic acids as postulated by Flax and Himes (*2*) can not entirely be excluded, the main determining factor of such a difference may reside in the "molecular" weight rather than in the molecular structure of nucleic acids.

It should further be pointed out that the findings presented in this paper may be of some importance in the physicochemical approach to the nature of metachromasia of synshetic dyes.

In this study the stoichiometry of the reaction between nucleic acids and thionin proved to be one dye molecule for each nucleic acid P, irrespective of different types of the latter. This is in good accord with classical data of Feulgen (*8*) using malachite green and crystal violet and with recent data of Herrman *el at.* (*9*) using toluidine blue. It differs greatly, however, from the one presented by Kurnick (*10, 11*) for the reaction between pDNA and methyl green (DNA P : dey=10 : 1). The intense stainability of histological preparations with thionin or toluidine blue as compared with methyl green seems to be due to the high binding ratio of the former dyes with nucleic acids.

SUMMARY

A spectrophotometric study on the *in vitro* metachromasia of thionin

caused by different types of nucleic acids showed that non-disaggregated DNA is more strongly metachromatic than PNA in either direction. Thus, both the blue color (α) taken at high molar rations of nucleic acid P to thionin and the reddish purple color (β) taken at lower ratios are more intensive with DNA than with PNA. The difference seems, at least in part, to be due to the difference in "molecular" weight of nucleic acids, since the intensity of metachromasia caused by disaggregated DNA is intermediate between PNA and non-disaggregated DNA. From the molar ratio of dye to nucleic acid P which gives the extreme β-color, the stoichiometry of the reaction was estimated to be one molecule of dye per nucleic acid P for every type of nucleic acid. The cytochemical implications of these findings were discussed briefly.

It is our pleasure to express our gratitude to Prof. Juntaro Kamahora of this department for his warm encouragement to this work.

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STUDIES ON HYDROLYTIC ENZYME OF NICOTINAMIDE

I. NICOTINAMIDASE OF BEER YEAST

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(Received for publication, October 5, 1953)

Since it was reported that nicotinamide is the component of pyridine nucleotides, coenzymes I and II, which are regarded as the components of dehydrogenases, playing an important part in the oxidation of living bodies, many attentions have been payed to the metabolism of nicotinamide.

Concerning deamination of the amide, Holman and De Lange (*1*) stated that the human body was capable of converting nicotinic acid into nicotinamide, but this change did not appear to be reversible. Ellinger and Abder Kader (*2*) described that when nicotinamide was administered *per os* to a rabbit or a guinea pig. the amide was deaminated and eliminated in urine as nicotinic acid, but they did not demonstrate in which organ of these animals the enzymatic deamination of nicotinamide occurred.

According to the reports of many investigators, some of microorganisms were also able to synthesize nicotinamide from nicotinic acid as the bacterial growth factor (*3–9*), but they did not mention in respect of converting nicotinamide into nicotinic acid.

In the following experiment the author has observed that the beer yeast has a sort of amidase activity which hydrolyses nicotinamide, evolving ammonia from the digest, and studied on some properties of this hydrolytic enzyme of the beer yeast.

On the other hand, it has been recognized that yeast has asparaginase activity as reported by Grassmann and Mayer (*10*), and Hiwatashi (*11*). As the properties of the beer yeast resembled in many points those of the yeast asparaginase, it was desirable to make a difference between nicotinamidase and asparaginase of the yeast. As there was found only an activity of the nicotinamidase in the autolysate of the pressed beer yeast with ethyl acetate it might be concluded that the nicotinamidase of the yeast differs from the yeast asparaginase.

EXPERIMENTAL

Method—The acetone powder of the beer yeast[1] was made 2 per cent homogenate in water, which was employed as the enzyme solution, and it was tested on its hydrolysing action on the nicotinamide[2] solution of 0.01 M final concentration at 37°. After incubation of certain period, certain amount of the digest solution was made alkaline with 1 ml. of saturated K_2CO_3 solution in the Conway's microdiffusion apparatus, and then ammonia diffused was caught by $N/150$ HCl solution. The residual acidity was titrated with $N/70$ NaOH solution, one drop of Tashiro's indicator being used. Here, 0.01 ml. of $N/70$ NaOH solution correspounds to 2 μg. of NH_3-N.

pH Optimum of Nicotinamidase of Acetone Powder of Beer Reast—Test solution contained 0.3 ml. of enzyme solution (2 per cent), 0.6 ml. of phosphate buffer of various pH values and 0.1 ml. of 0.1 M nicotinamide solution. Blanks consisted of 0.3 ml. of enzyme solution, 0.6 ml. of phosphate buffer, and 0.1 ml. of water without substrate. The digest solutions of pH 5.0 and 9.0 were prepared by adjusting the pH with diluted HCl and NaOH solution, respectively.

These digest solutions were incubated under toluene at 37° for 24 hours, and ammonia evolved in 0.6 ml. of the test or blank solution was determined by the Conway's method. The percentage of the values of enzymatic hydrolysis for that of the total hydrolysis calculated, is given in the parenthesis in tables.

Control tests were carried out under the same conditions with substrate and buffer without enzyme, but no ammonia formation was ascertained in all control solutions. The results are shown in Table I.

Effect of Heat on Nicotinamidase of Reast Powder—A part of 2 per cent homogenate of the beer yeast powder was heated for 15 minutes in a boiling water-bath and this heated enzyme solution was examined on its enzymatic activity.

Test solutions consisted of 0.6ml. of the enzyme solution heated, 1.2ml. of phosphate buffer (pH 7.5) and 0.2 ml. of 0.1 M nicotinamide solution. Control solutions contained the enzyme solution without heating. Blanks without substrate were examined.

After incubation, ammonia evolved in 0.6 ml. of the test digest was estimated as described in the above experiment. The values corrected for blanks are given in Table II.

Nicotinamiease of the Centifuged Homogenate of Reast Acetone Powder—Enzyme solution was prepared by centrifuging the homogenate containing the yeast powder in 2 per cent concentration. The supernatant was employed as enzyme A, and the precipitate, which was suspended in the same quantity of water as original volume, was used as enzyme B.

Test solution contained 0.6 ml. of enzyme A or B was used in these experiments.

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[1] Here the author intends to express many thanks to the Asahi Brewery Company for the kind delivery of the yeast.

[2] Preparation of the Takeda Pharmaceutical Industries Company.

TABLE I

pH Optimum of Nicotinamidase of Acetone Powder of Beer Yeast

Amonia evolved in 0.6 ml. of digest ($\mu g.$ NH_3-N)							
pH							
5.0	6.0	6.5	7.0	7.5	8.0	8.5	9.0
21.8	32.2	38.4	41.2	42.6	38.8	33.2	23.9
(25.9%)	(38.5%)	(45.7%)	(49.0%)	(50.7%)	(46.2%)	(39.5%)	(27.6%)

TABLE II

Effect of Heat on Nicotinamidase of Acetone Powder of Yeast

Ammonia evolved in 0.6 ml. of digest ($\mu g.$ NH_3-N)		
Incubation (*hrs.*)	Enzyme	
	Heated	Not heated
24	0.2 (0.2%)	42.6 (50.7%)
48	0.3 (0.3%)	75.6 (90.0%)

TABLE III

Nicotinamidase of the Centrifuged Homogenate

Ammonia evolved in 0.6 ml. of digest ($\mu g.$ NH_3-N)		
Incubation (*hrs.*)	Enzyme A (supernatant)	Enzyme B (precipitate)
24	5.6 (6.6%)	33.6 (40.0%)
48	11.2 (13.3%)	61.0 (72.6%)

Blanks consisted of 0.2 ml. of water instead of the substrate. The results are given in Table III.

Inhibiting Effect of Metal Salts and Enzyme Chemicals on Nicotinamidase—Test solution consisted of 0.6 ml. of enzyme (2 per cent homogenate), 0.2 ml. of metal salt or enzyme chemical solution, 1.0 ml. of phosphate buffer (pH 7), and 0.2 ml. of 0.1 *M* nicotinamide solution. The final concentrations of these effectors were as indicated in the following Tables IV and V. Controls contained all components except 0.2 ml. of metal salt or enzyme chemical solution. Blanks contained a) 0.6 ml. of enzyme, 0.2 ml. of effector

TABLE IV

Inhibiting Effect of Metal Salts on Nicotinamidase

Ammonia evolved in 0.6 ml. of digest ($\mu g.$ NH_3-N)

Metal salts (0.01 *M*)	Incubation (*hrs.*)	
	24	48
Control	42.6 (50.7%)	64.8 (77.1%)
$HgSO_4$	1.4 (1.6%)	2.2 (2.6%)
$CuCl_2$	8.4 (10.0%)	9.0 (10.7%)
$CuSO_4$	9.8 (11.6%)	9.4 (11.2%)
$FeSO_4$	32.8 (39.0%)	43.6 (51.9%)
$CoCl_2$	34.4 (40.9%)	57.0 (67.8%)
$MnCl_2$	38.2 (45.4%)	61.4 (73.1%)

TABLE V

Effect of Enzyme Chemicals on Nicotinamidase

Ammonia evolved in 0.6 ml. of digest ($\mu g.$ NH_3-N)

Enzyme chemicals	Final concentration (*M*)	Incubation (*hrs.*)	
		24	48
NaF	0	42.6 (50.7%)	64.6 (76.9%)
,,	0.01	42.2 (50.6%)	65.0 (77.3%)
,,	0.02	42.8 (50.9%)	64.0 (76.2%)
NaFHF	0	44.0 (52.4%)	67.6 (80.4%)
,,	0.01	44.2 (52.6%)	64.4 (76.6%)
,,	0.02	43.8 (52.1%)	65.6 (78.1%)
CH_2ICOOH	0	42.4 (50.4%)	65.2 (77.6%)
,,	0.01	40.8 (48.5%)	61.8 (73.5%)
KCN	0	41.6 (49.5%)	61.6 (73.3%)
,,	0.01	40.2 (47.8%)	58.4 (69.7%)
,,	0.02	39.0 (46.4%)	56.8 (67.6%)
Cysteine	0	44.0 (52.3%)	62.6 (74.5%)
,,	0.01	44.0 (52.3%)	59.4 (70.8%)
,,	0.02	46.4 (55.2%)	60.6 (72.1%)
H_2S	0	46.0 (54.7%)	66.1 (78.1%)
,,	0.0001	44.4 (52.8%)	64.3 (76.6%)
,,	0.01	42.4 (50.5%)	64.0 (76.2%)

solution, 1.0 ml. of buffer, and 0.2 ml. of water without substrate, b) 0.6 ml. of enzyme, 1.0 ml. of buffer and 0.4 ml. of water without both substrate and effector solutions.

TABLE VI

Amidase Activities of Yeast Powder

Ammonia evolved in 0.6 ml. of digest ($\mu g.$ NH_3-N)		
Substrates (0.01 *M*)	Incubation (*hrs.*)	
	24	48
Nicotinamide	42.6 (50.7%)	65.2 (77.6%)
Asparagine	49.4 (48.1%)	52.4 (61.2%)
Benzamide	0	0
Acetamide	0	0.8 (0.9%)

TABLE VII

Nicotinamidase and Asparaginase of Animal Tissues

Ammonia evolved in 0.6 ml. of digest ($\mu g.$ NH_3-N)							
Substrates	Incubation (*hrs.*)	Pig		Rabbit		Dog	
		Liver	Kidney	Liver	Kidney	Liver	Kidney
Nicotinamide	24	3.8 (4.5%)	3.2 (3.8%)	2.2 (2.6%)	4.0 (4.7%)	0.4 (0.4%)	2.8 (3.3%)
	48	—	3.8 (4.5%)	5.2 (6.2%)	5.1 (6.1%)	0.6 (0.7%)	3.1 (3.7%)
Asparagine	24	—	40.4 (48.1%)	44.8 (53.3%)	6.5 (7.7%)	25.2 (30.0%)	5.4 (6.4%)
	48	—	52.8 (62.8%)	53.2 (63.3%)	16.2 (19.8%)	40.2 (47.8%)	—

These solutions, adjusted to pH 7.0, were incubated under toluene at 37°. The values of ammonia produced are corrected for blanks and given in Tables IV and V.

Behaviours of Asparagine, Benzamide, and Acetamide towards Beer Yeast Powder—Amidase activity of the yeast powder was investigated on asparagine, benzamide, and acetamide besides nicotinamide. Tests and blanks were carried out under the same conditions as previous experiments. The results are illustrated in Table VI.

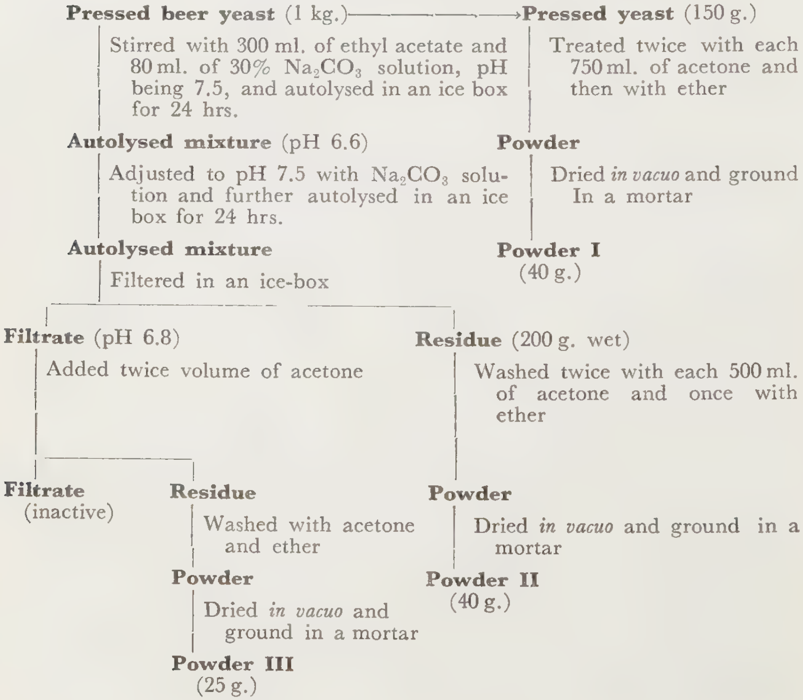
Behaviour of Nicotinamide towards Homogenate of Animal Tissues—From the experiments mentioned above it was evident that nicotinamide was hydrolysed by the beer yeast powder, so that the behaviour of nicotinamide towards animal tissues was further investigated. As the beer yeast powder which attacks nicotinamide showed also asparginase activity (Table VI), the liver or kidney of animals, having high activity of asparaginase, has been examined for its action upon nicotinamide.

Enzyme solution was prepared by homogenizing 1 g. of animal tissues in 4 ml. of water and it was prepared whenever used. Test solutions contained 0.8 ml. of enzyme solution, 1.0 ml. of phosphate buffer (pH 7.5), and 0.2 ml. of 0.1 M nicotinamide solution. Blanks consisted of 0.8 ml. of enzyme, 1.0 ml. of buffer, and 0.2 ml. of water instead of substrate.

After the incubation period of 24 and 48 hours under toluene at 37°, ammonia evolved in 0.6 ml. of the test digest was determined. The results are shown in Table VII.

Separation of Nicotinamidase from Asparaginase of Beer Yeast Powder—From Table VI it was apparent that the beer yeast powder has the activity of asparaginase as well as nicotinamidase. Therefore the author has here tried to distinguish both enzymes, and succeeded to prepare a partially purified sample of enzyme, which was able to hydrolyse remarkably nicotinamide but not asparagine. The procedures of the separation are shown in the following Scheme 1.

SCHEME 1. Separation of yeast nicotinamidase from its asparaginase


Pressed beer yeast (1 kg.)
Pressed yeast (150 g.)
Stirred with 300 ml. of ethyl acetate and 80 ml. of 30% Na_2CO_3 solution, pH being 7.5, and autolysed in an ice box for 24 hrs.
Treated twice with each 750 ml. of acetone and then with ether
Autolysed mixture (pH 6.6)
Powder
Adjusted to pH 7.5 with Na_2CO_3 solution and further autolysed in an ice box for 24 hrs.
Dried *in vacuo* and ground In a mortar
Autolysed mixture
Powder I (40 g.)
Filtered in an ice-box
Filtrate (pH 6.8)
Residue (200 g. wet)
Added twice volume of acetone
Washed twice with each 500 ml. of acetone and once with ether
Filtrate (inactive)
Residue
Powder
Washed with acetone and ether
Dried *in vacuo* and ground in a mortar
Powder
Powder II (40 g.)
Dried *in vacuo* and ground in a mortar
Powder III (25 g.)

Nicotinamidase and Asparaginase Action of Powders I, II, and III—Enzyme solutions were prepared by suspending 100 mg. of powder I (direct acetone powder), 50 mg. of powder II (autolysed residue), and 50 mg. of powder III (autolysate) in each 5 ml. of water. They were prepared every time used.

Test solutions contained 0.15 ml. of 0.1 *M* nicotinamide or 0.1 *M* asparagine solution, 0.6 ml. of enzyme solution, and 0.75 ml. of phosphate buffer (pH 7.5). Blanks contained 0.6 ml. enzyme, 0.75 ml. of buffer, and 0.15 ml. of water instead of substrate.

These test and blank solutions were incubated at 37° for 3 and 5 hours. After the reaction ammonia produced in 0.6 ml. of each solution was determined, and the values, corrected for blanks, are given in Table VIII.

TABLE VIII

Nicotinamidase and Asparaginase Action of Powers I, II, and III

Ammonia evolved in 0.6 ml. of digest ($\mu g.$ NH_3-N)

Enzyme preparation	Substrate			
	Nicotinamide		Asparagine	
	3 hrs.	*5 hrs.*	*3 hrs.*	*5 hrs.*
Powder I	16.6 (19.7%)	23.4 (27.8%)	33.2 (39.5%)	47.0 (55.9%)
Powder II	7.2 (8.5%)	13.0 (15.4%)	4.0 (4.7%)	5.8 (7.0%)
Powder III	39.2 (46.6%)	54.2 (64.3%)	0	0

Then, total nitrogen of these powders I, II and III was estimated by the Kjeldahl-Nessler method (*12*) in order to compare the activities of nicotinamidase per mg. of enzyme nitrogen. The results obtained are shown in Table IX.

pH Ompimum of nicotinamidase of Powder III (*Autolysate*)—100 mg. of powder III was suspended in 10 ml. of water, and it was used as enzyme solution. Test solution

TABLE IX

Nicotinamidase Activity of Powders I, II, and III

Enzyme preparations	$\mu g.$ NH_3-N/mg. enzyme N/hr.	Ratio
Powder I*	6.1	1
Powder II+	5.1	0.8
Powder III*	24.3	4.0

Enzyme nitrogen was estimated for: * 0.5 ml. of 5-fold dilution of enzyme solution; + 0.25 ml. of 2-fold dilution of enzyme solution.

TABLE X

pH Optimum of Nicotinamidase of Powder III

Ammonia evolved in 0.6 ml. of digest (*μg.* NH_3-N)

pH 6.0	6.5	7.0	7.5	8.0	8.5
26.3 (31.3%)	45.2 (54.2%)	62.6 (74.5%)	71.8 (85.4%)	67.0 (79.7%)	55.7 (66.4%)

and blanks were similarly prepared as in the above experiments and incubated at 37° for 5 hours. The results are given in Table X.

Inhibiting Effect of Metal Salts and Enzyme Chemicals on Nicotinamidase of Powder III —Enzyme solution was prepared by suspending 50 mg. of powder III in 5 ml. of water Test solutions consisted o, 0.15 ml. of 0.1 *M* nicotinamide, 0.6 ml. of enzyme, 0.6 ml.

TABLE XI

Inhibiting Effect of Metal Salts and Enzyme Chemicals on Nicotinamidase of Powder III

Ammonia evolved in 0.6 ml. of digest (*μg.* NH_3-N)

Effectors (0.01 *M*)	Incubation (*hrs.*) 3	5
Control	51.6 (61.4%)	74.6 (88.8%)
$HgSO_4$	0	0
$HgSO_4$*	2.2 (2.6%)	3.4 (4.1%)
$CuSO_4$	19.4 (23.1%)	33.0 (39.2%)
$CuCl_2$	20.0 (23.8%)	35.6 (42.3%)
$FeSO_4$	24.4 (29.0%)	51.8 (61.6%)
$CoCl_2$	45.8 (54.5%)	56.8 (67.6%)
$MnCl_2$	49.0 (58.3%)	62.0 (73.8%)
$MgCl_2$	40.2 (47.8%)	70.6 (84.0%)
KCN	50.0 (59.5%)	75.2 (89.5%)
Cysteine	49.4 (58.8%)	76.6 (91.2%)
CH_2ICOOH	52.8 (62.8%)	76.0 (90.4%)
NaF	49.2 (58.5%)	74.8 (89.0%)
NaFHF	53.4 (64.2%)	72.8 (86.6%)

* Final concentration was 0.001 *M*.

of phosphate buffer (pH 7.0), and 0.15 ml. of 0.1 *M* metal salt or enzyme chemical solution, the final concentration of which corresponded to 0.01 *M* solution. Controls without effector solution were examined under the same conditions. Blanks only without substrate or without both substrate and effector were carried out.

After incubation at 37°, ammonia produced was determined and the values corrected for blanks are illustrated in Table XI.

RESULTS AND CONSIDERATION

The pH optimum of the nicotinamidase of the beer yeast powder seems to lie between 7.0 and 7.5 (Table I). This comparatively resumbles the pH optimum 7.5 of the yeast asparaginase reported by Hiwatashi (*11*).

Nicotinamidase was completely lost by heating at 100° for 15 minutes (Table II). When the homogenate of the yeast powder was centrifuged, the precipitate was more active than the supernatant and their activity ratio is 6 : 1 (Table III). It seems probable, therefore, that treating with acetone caused denaturation of the cell membrane of yeast and consequently inhibition of eluting the enzyme into fluid.

In the experiments of effect of metals, nicotinamidase was inhibited highly by Hg^{++}, next by Cu^{++}, slightly by Fe^{++} and Co^{++}, and not by Mn^{++} under the conditions employed (Table IV). The effect of enzyme chemicals on nicotinamidase was scarcely found in any case at the concentration of 0.01 *M* and 0.02 *M*, though rather an inhibitory tendency showed by KCN on the amidase action in its higher concentration seems to be similar to its effect on the yeast asparaginase which was inhibited at high concentration of KCN (*10*) (Table V).

A similar degree of splitting value was found in both cases of nicotinamide and asparagine at the same concentration (Table VI). It will be seen from Table VI that acetone powder of the yeast has both asparaginase and nicotinamidase activities, but neither acetamidase nor banzamidase.

In the experiments for animal tissues, almost all of livers and kidneys examined, with exception of dog liver and pig liver, showed a high asparaginase activity. On the contrary, nicotinamidase activity remained in such a grade as low as less than 6 per cent in any case (Table VII). In this respect, asparaginase appears to be different from nicotinamidase in animal tissues, contrary to the yeast powder which has both strong enzyme activities, asparaginase and nicotinamidase. Nishio (*13*) reported that deamination of some amino acids by liver or kidney of cow and pig was accelerated by the addition of riboflavin. Hence, experiments for the nicotinamidase of animal tissues were carried out in the presence of riboflavin, but no effect was observed. In another experiment, it was observed that the addition of heated yeast preparation could not also accelerate the action of animal tissue homogenates on nicotinamide.

While preparing this paper, there was an opportunity to review the abstract of Quagliariello *et al.* (*14*), in which it was described that nicotinamide was synthesized and hydrolysed by the slice or hemogenate of rat tissues, and nicotinic acid or nicotinamide produced was confirmed by the paper chromatographic procedure. As for the animal enzymes further experiments are to be carried out in this laboratory.

The author has succeeded in the preparation of the yeast nicotinaamidase free from asparaginase by autolysing beer yeast with ethyl acetate (Scheme 1). The supernatant fluid of the autolysate showed only a nicotinamidase action but not asparaginase (Table VIII). The yeast nicotinamidase separated from asparaginase showed an increased activity four times as strong as the original yeast powder (Table IX). It will be seen from Tables X and XI that the pH optimum of this purified enzyme preparation and its behaviour towards metal salts and enzyme chemicals are almost similar to those of the acetone powder containing asparaginase.

SUMMARY

1. The acetone powder of the beer yeast has an amidase activity which hydrolyses nicotinamide and asparagine, evolving ammonia, but neither acetamide nor benzamide. Its pH optimum seems to lie between 7 and 7.5.

2. A partially purified enzyme preparation, containing a nicotinamidase activity but not asparaginase, was resulted by autolysing the raw pressed beer yeast with ethyl acetate.

3. Effect of metal salts and enzyme chemicals on nicotinamidase of the beer yeast was investigated. $HgSO_4$ inhibited the amidase severely, $CuSO_4$ and $CuCl_2$ notably, $FeSO_4$ and $CoCl_2$ weakly, but $MnCl_2$ showed no effect. Influence of KCN, cysteine, H_2S, NaF, NaFHF, and CH_2ICOOH was hardly recognized under the conditions employed.

4. Animal tissues such as liver and kidney showed a strong asparaginase activity but a minimal action of nicotinamidase.

The author wishes to express his deep gratitude to Prof. Dr. Senji Utzino for his constant guidance in the course of this work.

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STUDIES ON HYDROLYTIC ENZYME OF NICOTINAMIDE

II. PURIFICATION OF NICOTINAMIDASE OF BEER YEAST

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In the previous paper (*1*), the author has reported that the beer yeast has an amidase activity which hydrolyses nicotinamide, evolving ammonia and that a sample of nicotinamidase was prepared from the raw pressed yeast by autolysing with ethyl acetate, which was about 2.5 times more active than the homogenate of the acetone powder obtained directly from the yeast.

The present communication is concerned with purification of this enzyme using of acetone fractionation or calcium phosphate gel adsorption procedure.

EXPERIMENTAL

A. Purification of Nicotinamidase of Yeast by Acetone Fractionation

The starting materials used through the experiments were the powder preparation III obtained by the method previously reported (*1*).

The amounts of acetone to be added in the procedure of acetone fractionation was calculated according to the following equation:

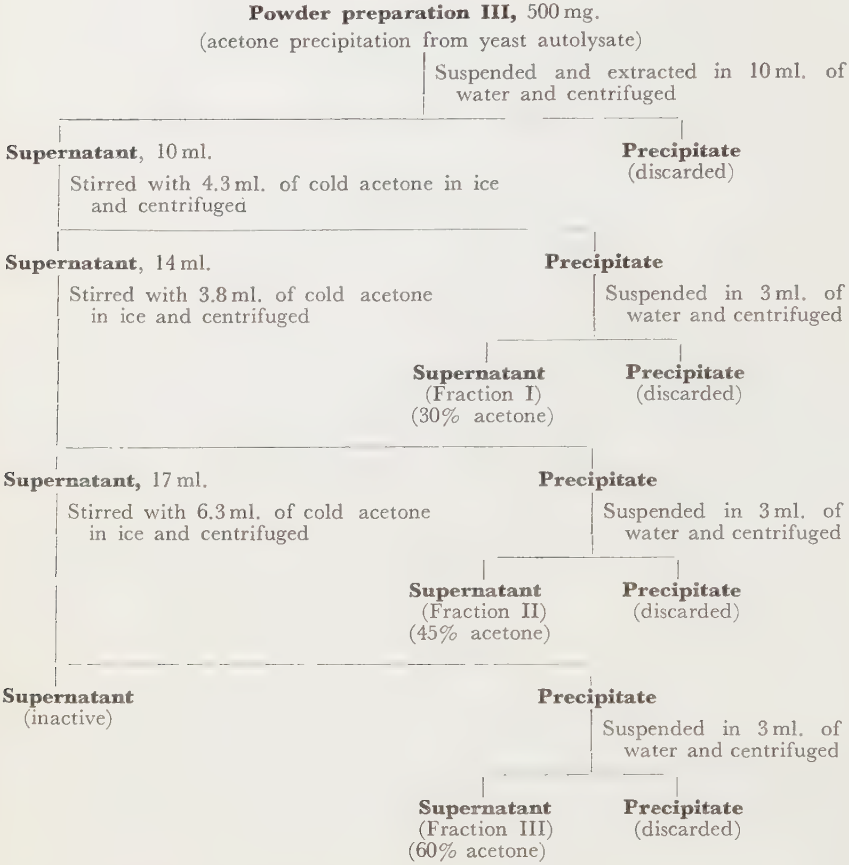
$$x = \frac{p(r-q)}{100-q}$$

where x is volume of acetone to be added (ml.), p volume of enzyme solution before each fractionation (ml.), q volume per cent of acetone in p solution, and r volume per cent of acetone wanted. The temperature was kept as low as possible (below 5°) during the acetone treatment.

The procedures of purification of the yearst nicotinamidase by acetone fractionation are shown in the following Scheme 1.

Nicotinamidase Activity of Each Acetone Fraction—For assay fractions I and III were employed without dilution, but fraction II with 2-fold dilution.

Test solutions consisted of 0.6 ml. of fraction I, II or III, 0.6 ml. of phosphate buffer (pH 7.5) and 0.15 ml. of 0.1 *M* nicotinamide solution. Blanks contained enzyme, Scheme 1. Purification of nicotinamidase by acetone fractionation


Powder preparation III, 500 mg.
(acetone precipitation from yeast autolysate)
Suspended and extracted in 10 ml. of water and centrifuged
Supernatant, 10 ml.
Precipitate (discarded)
Stirred with 4.3 ml. of cold acetone in ice and centrifuged
Supernatant, 14 ml.
Precipitate
Stirred with 3.8 ml. of cold acetone in ice and centrifuged
Suspended in 3 ml. of water and centrifuged
Supernatant (Fraction I) (30% acetone)
Precipitate (discarded)
Supernatant, 17 ml.
Precipitate
Stirred with 6.3 ml. of cold acetone in ice and centrifuged
Suspended in 3 ml. of water and centrifuged
Supernatant (Fraction II) (45% acetone)
Precipitate (discarded)
Supernatant (inactive)
Precipitate
Suspended in 3 ml. of water and centrifuged
Supernatant (Fraction III) (60% acetone)
Precipitate (discarded)

buffer solution and 0.15 ml. of water without substrate.

These test and blank solutions were incubated for 1 hour and 2 hours at 37°. To stop the enzyme action, 0.15 ml. of $4N$ H_2SO_4 was added to the digest and ammonia evolved in 1.0 ml. of each digest was estimated by the Conway's method as in the previous report (*1*). The values of the test solutions, corrected for blanks, are given in Table I.

For the purpose of comparing the enzyme activities the total protein nitrogen of the enzyme solution of the three fractions and the original powder was estimated by the Kjeldahl-Nessler method (*2*), and the ammonia formation was compared on the basis of mg. total nitrogen of these preparations. The comparative values are also given in Table I as rate of μg. NH_3-N per mg. enzyme-N per hour.

TABLE I

Nicotinamidase Activity of the Acetone Fractions

Enzyme preparation	NH_3-N evolved*		Rate†	Ratio
	1 hr.	*2 hrs.*		
Original powder III††	14.0	29.0	23.4	1.0
Fraction I (30%)	6.0	12.2	20.8	0.9
Fraction II (45%)	40.2	80.0	58.0	2.5
Fraction III (60%)	10.6	19.8	11.9	0.5

* μg. NH_3-H evolved in 1.0 ml. of digest.

† μg. NH_3-N evolved per mg. enzyme nitrogen per hour.

†† 60 mg. of powder III was suspended in 3 ml. of water. After centrifugation, the supernatant was employed as enzyme solution. Experimental conditions were the same as used foı fraction I–III.

B. Purification of nicotinamidase by calcium phosphate gel adsorption

The procedures of purification of the yeast nicotinamidase by adsorption on 0.1 volume per cent calcium phosphate gel are given in Scheme 2. The powder preparation III (*1*) employed as the starting material in this experiment too.

Nicotinamidase Activity of Each Supernatant after Acid Treatment of Adsorption and Eluation by Means of $Ca_3(PO_4)_2$-gel Treatment—Test solution consisted of 0.6 ml. of supernatant I, II, III or IV, 0.6 ml. of phosphate buffer (pH 7.5) and 0.15 ml. of 0.1 *M* nicotinamide. Blanks were composed of enzyme, buffer solution and 0.15 ml. of water without substrate.

After incubation under the same conditions, enzyme action was interrupted by the addition of 0.15 ml. of 4 *N* H_2SO_4 and ammonia evolved in 1.0 ml. of the test digest was determined. The values, corrected for blanks, are given in Table II.

Rates of ammonia formation per mg. total nitrogen of the enzyme solutions are also given in Table II.

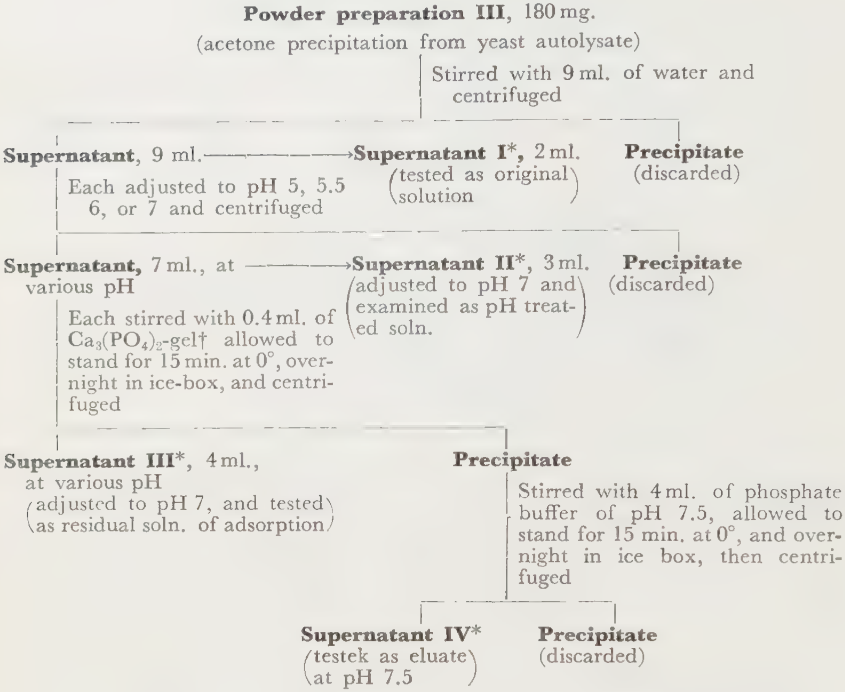
Among the results of Table I and II, the rate of the supernatant I (powder III), fraction II, or supernatant IV per mg. enzyme nitrogen per hour was compared with that of the acetone powder I (*1*), and it is given in Table III with their ratio.

RESULTS AND CONSIDERATIONS

As shown in Table I, the acetone fraction of its 45 volume per cent is the highest in the nicotinamidase activity, being 2.5 times more active (on the basis of powder nitrogen) than the original acetone powder (powder III), while the other two fractions (fractions I and III) remained only slightly active.

From Table II, it is clear that the eluate (supernatant IV) obtained

SCHEME 2. Purification of nicotinamidase by calcium phosphate gel adsorption


Powder preparation III, 180 mg.
(acetone precipitation from yeast autolysate)
Stirred with 9 ml. of water and centrifuged
Supernatant, 9 ml.
Supernatant I*, 2 ml.
(tested as original solution)
Precipitate
(discarded)
Each adjusted to pH 5, 5.5 6, or 7 and centrifuged
Supernatant, 7 ml., at various pH
Supernatant II*, 3 ml.
(adjusted to pH 7 and examined as pH treated soln.)
Precipitate
(discarded)
Each stirred with 0.4 ml. of Ca3(PO4)2-gel† allowed to stand for 15 min. at 0°, overnight in ice-box, and centrifuged
Supernatant III*, 4 ml., at various pH
(adjusted to pH 7, and tested as residual soln. of adsorption)
Precipitate
Stirred with 4 ml. of phosphate buffer of pH 7.5, allowed to stand for 15 min. at 0°, and overnight in ice box, then centrifuged
Supernatant IV*
(testek as eluate at pH 7.5)
Precipitate
(discarded)

* Supernatant I and II were used for assay as enzyme solutions on the day when they were prepared and supernatant III and IV on the next day.

† $Ca_3(PO_4)_2$-gel was prepared by the method of Kunitz (3).

by calcium phosphate gel adsorption at pH 6.0 shows the strongest activity of the yeast nicotinamidase, while the residual fluid of the adsorption remained almost always to be the weakest in its activity. In addition, it seems to be more suitable to use 0.1 volume per cent calcium phosphate gel than 0.2 volume per cent in the adsorption procedure for purification of the enzyme.

It is acceptable from Table III that the supernatant IV, the eluate obtained from the $Ca_3(PO_4)_2$-gel adsorbate with phosphate buffer of pH 7 possesses the activity 4 times stronger than that of the powder preparation III precipitated from the autolyzed yeast with ethyl acetate, and it has also higher activity than the acetone fraction II in 45 per cent.

Table II

Nicotinamidse Activity of the Supernatants Obtained bf Adsorption Procedure at Various PH

pH of adsorption		Supernatant I (original solution)		Supernatant II (pH treated solution)		Supernatant III (adsorption residue)		Supernatant IV (eluate)	
		1 hr.	2 hrs.	1 hr.	2 hrs.	1 hr.	2 hrs.	1 hr.	2 hrs.
5.0	NH_4-N* evolved	15.2	—	8.0	15.8	2.0	3.8	2.6	5.2
	Rate	24.0		18.1		6.0		29.3	
	Ratio	1.0		0.8		0.3		1.2	
5.5	NH_3-N evolved	15.6	—	10.8	23.2	2.0	3.2	7.2	14.6
	Rate	24.3		21.6		5.7		76.2	
	Ratio	1.0		0.9		0.2		3.1	
6.0	NH_3-N evolved	15.8	—	12.0	25.4	4.2	8.0	8.0	15.0
	Rate	25.5		24.2		5.2		96.2	
	Ratio	1.0		1.0		0.2		3.8	
6.0†	NH_3-N evolved	14.2	—	14.2	29.0	1.8	2.8	7.2	14.2
	Rate	23.5		27.1		5.4		84.1	
	Ratio	1.0		1.2		0.2		3.6	
7.0	NH_3-N evolved	15.2	—	—	—	3.2	6.2	6.8	13.6
	Rate	23.1				8.9		67.8	
	Ratio	1.0				0.4		3.0	

* μg. NH_3-N evolved in 1.0 ml. of digest.

† The data were obtained when 0.2 volume per cent $Ca_3(PO_4)_2$-gel was added instead of 0.1 volume per cent gel.

SUMMARY

A purification of the beer yeast nicotinamidase was tried by the procedures of acetone fractionation or calcium phosphate gel adsorption.

1. By the procedure of the acetone fractionation in 45 per cent a partially purified enzyme was prepared, which was about 2.5 times more active (on the basis of enzyme nitrogen) than the starting material and 4 times more active than the original acetone powder of the beer yeast.

TABLE III

Comparison of Nicotinamidase Activities of Four Yeast Preparations

Preparation	μg. NH_3-N/mg. enzyme N/hr.	Ratio	
Acetone powder I	6.2*	1.0	
Supernatant I (original solution from powder prepn. III before adsorptiln)	24.0†	3.9	1.0
45% acetone fraction (Table II)	58.0	9.4	2.4
Supernatant IV (eluate from $Ca_3(PO_4)_2$-gel adsorption)	96.2	15.6	4.0

* Enzyme solution: 160 mg. of the acetone powder I (1) was suspended in 4 ml. of water and used as enzyme source. The total nitrogen of the enzyme protein was estimated for 0.25 ml. of this enzyme solution diluted 1 : 5.

† Average of the values of the original solution in the third column in Table II.

2. Using the adsorption procedure with calcium phosphate gel at pH 6.0, considerable activity of this enzyme was found in the portion of the eluate, which is 4 times and 15 times more active than the starting material and the original acetone powder, respectively.

The author wishes to express his thanks to Prof. Dr. Senji Utzino for suggesting this investigation as well as for constant guidance in the course of the work.

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ENZYMIC DEGRADATION OF D-GLUCOSEAMINE BY ESCHERICHIA COLI

I. THE MECHANISM OF BREAKDOWN

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Enzymological studies on the decomposition of glucosamine, by washed suspensions of resting bacteria, have been commenced relatively recently, though many classical investigators (see *e.g.* Kawakami (*1*)) had noted disappearance of glucosamine from the medium along with the formation of ammonia and other substances by growing organisms. In 1941, Lutwak-Mann (*2*) studied the metabolism of amino sugars by enzymes of mammalian tissues and added some preliminary reports on enzymes of yeast and of bacteria. She pointed out that washed suspensions of *Bacterium coli*, *Streptococcus faecalis* and *Proteus vulgaris* decomposed glucosamine either aerobically or anaerobically with formation of ammonia and other unidentified products. She also observed that the addition of glucose resulted in strong inhibition of ammonia formation, while potassium cyanide strongly depressed the oxidation. Rosenberg (*3*) investigated effects of imidazole buffer and of sodium azide on the glucosamine decomposition by washed suspensions of *Escherichia coli*, and stated that two ways might exist for the aerobic degradation, either through or not through phosphorylation process, while there is only one or phosphorylating course of the anaerobic decomposition, Rogers (*4*) found that washed suspensions of *Strep. haemolyticus* and *Strep. viridans* decomposed 1 mole of N-acetylglucosamine with formation of 1 mole of ammonia, 1.2–1.7 moles of lactate and 1 equivalent of volatile acid.

With respect to the mechanism of degradation, Lutwak-Mann claimed from the results obtained with mammalian tissues that the oxidation preceded deamination, and this was commented as "most likely hypothesis" by Rogers; with *Esch. coli* as enzyme, however, little has been so far discussed concerning this point.

The present studies have been undertaken in order to clarify the mechanism of glucosamine decomposition by *Esch. coli*, washed suspensions of bacteria being used as enzyme and D-glucosamine as substrate. The data obtained seem to suggest that both aerobic oxidation and anaerobic gas production—probably hydrogen—belong to different steps of degradation from the ammonia formation, and that the deamination takes place prior to oxidation or hydrogen production.

EXPERIMENTAL

Bacteriological—Escherichia coli K_{12} was obtained from the Research Institute for Microbial Diseases, Osaka University, and maintained on broth agar slants before inoculation. The medium used in this study consisted of 3 per cent peptone, 2 per cent glucose, and 0.5 per cent NaCl solution, glucose being added to stimulate enzyme formation (*5*). 200–300 ml. of the medium was transferred in 500-ml. Erlenmeyer flask, and autoclaved under 7 lbs. pressure for 10 minutes. Initial pH was 5.8 and the final one 4.7–4.8. Bacteria were cultivated at 37° for 18 hours without agitation and harvested by centrifugation.

Enzyme Preparation—The harvested cells were washed twice with 0.9 per cent NaCl solution, collected by centrifugation, and then suspended in 0.9 per cent NaCl solution in such a concentration that the suspension contains about 1 mg. bacterial nitrogen per ml. The fresh suspension was consistently used as enzyme solution in this study, except in the study on the decay of enzyme activity on standing.

Standard Method—A standard Warburg flask of about 15-ml. capacity was used. To the main chamber of the flask were added 0.6 ml. of $M/15$ phosphate buffer at pH 7.2 and 0.6 ml. of enzyme solution, and to the side-arm 0.15 ml. of neutralized $M/10$ solution of substrate; in addition to these, 0.15 ml. of neutralized solution of effectors or water was added to make up total volume 1.5 ml. To absorb carbon dioxide evolved, if any, during the reaction, 0.2 ml. of 10 per cent KOH with a piece of filter paper was placed in the center well of the flask. Gas phase was filled with air. After 15-minutes' equilibration at 37°, the substrate was tipped quantitatively into the main chamber, the gas change being noted for every 10 minutes up to one hour, when the reaction was stopped by the addition of 0.25 ml. of $4N\ H_2SO_4$ and then analysis on ammonia was made for suitable aliquot (0.7–1.4 ml.) of the final mixture by the microdiffusion method of Conway (*6*).

Special Techniques—For anaerobic studies of enzyme reaction, the air in gas phase of Warburg apparatus was replaced by nitrogen or carbon monoxide, treated with alkaline pyrogallol solution, by means of evacuation procedure described by Umbreit *et al.* (*7*). The same procedure was also employed to fill up flasks with Air-O_2 (or -N_2) mixtures of various ratios. In experiments with potassium cyanide, 0,6 ml. of Ca$(CN-)_2$-Ca$(OH)_2$ or KCN-KOH mixtures described by Robbie (*8*) was added in the center well of the flask instead of usual 0.2 ml. of 10 per cent KOH, so that HCN tension may be maintained constant throughout the reaction period.

Chemical Preparations—D-Glucosamine hydrochloride was prepared by hydrolyzing crab shells with HCl as described by Purchase and Braun (*9*) and used in this study after recrystallizing several times. Adenosine triphosphate (ATP) separated as dibarium salt from the rabbit muscle by usual method was converted into sodium salt according to the method described by Lepage (*10*).

Bacterial Nitrogen—Total Nitrogen of enzyme solution was estimated for 0.24 ml. aliquot by Kjeldahl-Nessler method with modification of Miller and Miller (*11*).

Symbols Indicating Enzyme Activities—Symbols used sn this communication are defined as follows:

$Q_{O_2}(N) = \mu l.$ O_2 taken up per mg. bacterial nitrogen per hour.
$Q_{NH_3}(N) = \mu l.$ NH_3 evolved per mg. bacterial nitrogen per hour.
$Q_{H_2}(N) = \mu l.$ H_2 given off per mg. bacterial nitrogen per hour.

In case necessary to indicate substrate definitely, it is added in parenthesis next to (N) as following example: $Q_{NH_3}(N)$(glucosamine). Under the conditions described in "Standard method," oxygen consumption and ammonia formation due to glucosamine breakdown showed following magnitudes in Q-values mentioned above:

$Q_{O_2}(N)$(glucosamine); Mean value (10 cases) $= 526 \pm 17.3$*
$Q_{NH_3}(N)$(glucosamine); Mean value (10 cases) $= 296 \pm 3.7$*
* Standard error of the mean.

As control, blank experiments with water in place of substrate were carried out under specified conditions, the results being:

$Q_{O_2}(N)$(endogenous) $= 20-30$; $Q_{NH_3}(N)$(endogenous) $= 5-10$.

The non-enzymic degradation of glucosamine under experimental conditions was also of negligible magnitude; therefore in this communication, the enzyme activities are shown without any correction for those of blank experiments.

RESULTS

Rates of Deamination and Oxidation of Glucosamine—Fig. 1 shows that both deamination and oxidation take place without appreciable lag. Amount of ammonia evolved under the conditions of "Standard method" reached about 75 per cent of the theoretical value in 90 minutes, and increased thereafter very slightly up to 150 minutes. In another experiment using twice concentrated suspension of bacteria, the amount of ammonia formation reached 75 per cent of the theoretical within 60 minutes, yet evolution did not exceed 80 per cent in 150 minutes. As shown in Fig. 1, oxygen consumption per 1 mole of glcosamine decomposed seembs to be 2.5–3.0 moles; this value is close to that obtained by Rosenberg (*3*) when adding azide (2.75 moles), but the same author reported that 1.5 moles of oxygen was taken up per 1 mole of glucosamine when omitting azide. It might be noted here that the magnitudes of $Q_{O_2}(N)$ are so variable depending upon conditions during bacterial growth (*5*) that one can hardly discuss the real implication of the absolute value of oxygen consumption.

Another point worthy of attention in Fig. 1 is the fact that the ammonia production reaches its maximal value before oxygen uptake ceases its steady increase: this may

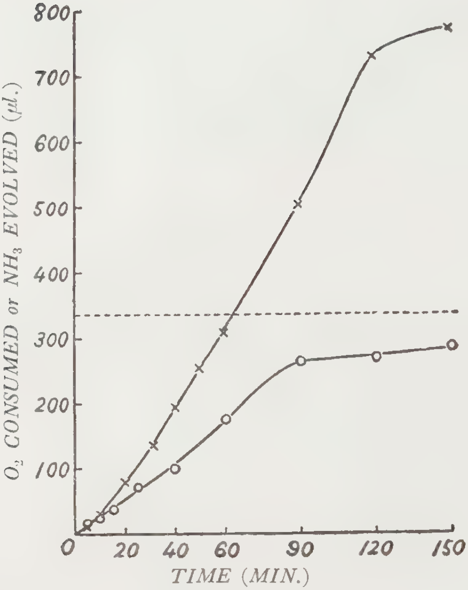

FIG. 1. Rate of deamination and oxidation of D-glucosamine by washed suspensions of *Esch. coli* K_{12}.

Conditions as in "Standard method." 0.25 ml. of H_2SO_4 was added in the second side-arm of a Warburg flask and tipped into main chamber in order to stop reaction just when desired; 0.6 ml. of enzyme solution contained 576 μg. of N in this case. ×—× μl. O_2 consumed, ○—○ μl. NH_3 evolved, ---- theoretical value for NH_3.

speak against Lutwak-Mann's observation (*2*) that oxidation preceded deamination in mammalian tissues.

pH Optimum for Deamination and Oxidation—The pH-activity curves in Fig. 2 show that under standard conditions both deamination and oxidation occur in maximal rates at pH value between 6.5 and 8.0. But in case the enzyme reaction proceeds in test tubes without agitation, the optimal pH is limited within the range of 7.0–7.5 as represented by another sharp curve (●—●) in Fig. 2. The pH optimum for amino sugar decomposition by mammalian tissues was reported to be 7.4–7.8 by Lutwak-Mann (*2*), while Rogers (*4*) stated it to lie betweee 7.0 and 7.5 for streptococci. The pH optimum observed by the present authors covers a range much broader than those reported by others.

Inhibition and Inactivation of the Enzyme—For the purpose of obtaining some indications as to the mechanism of reaction involved in the enzymic degradation of

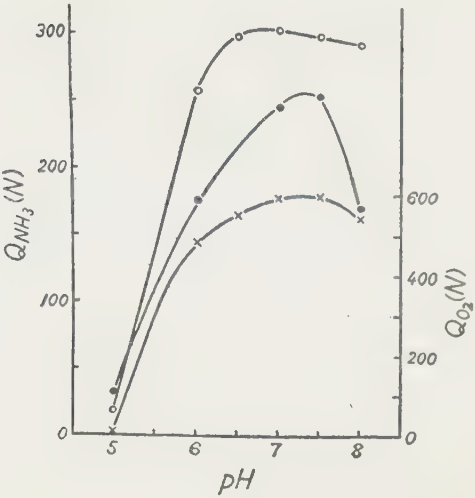

FIG. 2. pH-activity curve in D-glucosamine degradation by washed suspensions of *Esch. coli* K_{12}.

M/15 phosphate buffer of varying pH was used. ○—○ Q_{NH_3} (N), ×—× Q_{O_2}(N), both employing "Standard method"; ●—● Q_{NH_3}(N), reaction was carried out in stoppered test tubes, instead of Warburg flasks, with same contents as in "Standard method" and without agitation at 37° for one hour.

glucosamine by *Esch. coli*, effects of several metabolic inhibitors were tested and the results are collected in Table I. This table contains also data showing the effect of heat or acetone treatment as well as the effect of such inhibitory agents on oxidation of glucose by the organisms. Table I shows the existence of parallelism, though not a complete one, between the case of glucosamine and that of glucose concerning the degree to which they are subject to inhibition of oxidation.

Monoiodoacetate and toluene, as reported by Lutwak-Mann (*2*), inhibited completely both deamination and oxidation. Toluence exhibited 100 per cent inhibition even by its vapour, and was also capable of immediately stopping the reaction already in progress. According to Rogers (*4*), when streptococci were used, complete inhibition could not be observed by merely saturating cell suspension with toluene. This kind of discrepancy in the effect of toluene may be related to the kinds of bacteria. Sodium fluoride (*M*/100) exhibited no effect on both deamination and oxidation, while fluosilicate inhibited strongly oxidation with slight depression of deamination. The effect of azide on the reaction was precisely investigated by Rosenberg (*3*) who claimed that addition of azide (*M*/250) resulted iu augmentation of oxygen consumption as well as of ammonia formation in the course of glucosamine degradation.

The present authors, however, observed that azide ($M/100$) inhibited oxidation only slightly with no effect on deamination. Lutwak-Mann stated that azide ($M/75$) exerted slight inhibitions on both oxidation and deamination in the enzyme system of kidney cortex.

TABLE I

Inhibition and Inactivation of Enzymic Degradation of D-*Glucosamine by Washed Suspensions of Esch. coli* K_{12}

(Conditions as in "Standard method," except where noted)

Inhibitor	Final concentration	Substrate		
		D-Glucosamine		D-Glucose
		$Q_{NH_3}(N)$	$Q_{O_2}(N)$	$Q_{O_2}(N)$
None		296	265	587
Monoiodoacetate	$M/100$	14	22	24
Toluene*	(a) added from the beginning	10	—	—
,,	(b) added after 30 min.	147	—	—
Sodium fluoride	$M/100$	304	545	560
Sodium fluosilicate	$M/100$	275	66	37
Sodium azide	$M/100$	298	449	366
Potassium cyanide	$M/100$	229	163	163
,,	$M/500$	306	186	163
,,	$M/5000$	328	244	186
sodium arsenite	$M/100$	262	177	341
,,	$M/500$	259	167	332
,,	$M/1000$	315	196	365
,,	$M/5000$	301	240	431
Heat treatment	50°, 10 min.	238†	—	—
	60°, 10 min.	5	—	—
Treatment with acetone‡	−5°, twice	6	—	—

* (a) 0.05 ml. of toluene added in the second side-arm of a Warburg flask and not tipped into the main chamber throughout the reaction; (b) 30 minutes after the substrate was tipped into main chamber containing enzyme solution, the Warburg flask was quickly removed from manometer, 0.05 ml. of toluene added to the mixture, flask set up again and shaken for another 30 minutes.

† Without agitation, in test tube; control experiment in test tube showed $Q_{NH_3}(N) = 230$.

‡ About 0.5 g. in wet weight of organism was treated twice with each 40 ml. of acetone and dried *in vacuo*; yield *ca.* 100 mg.

Upon reviewing the effect of potassium cyanide and sodium arsenite in Table I, it will be noticed that the oxidation is affected by these inhibitors in such low concentrations that no change occurs in deamination, while the latter comes to be inhibited slightly only in their high concentrations. That the deamination remains unchanged whereas the oxidation is depressed held also for fluosilicate mentioned above, and this series of facts might offer a key to understanding of the mechanism of enzymic degradation of glucosamine.

Table I also shows that enzymic activity of the washed suspension is not inactivated by the application of heat at 50° for 10 minutes, but completely destroyed by heat treatment at 60° or by acetone treatment.

Effect of Carbohydrates on the Reaction—Lutwak-Mann (*2*) found the inhibitory effect of glucose on enzymic decomposition of glucosamine, and the similar result was obtained by Rogers (*4*) with streptococci. The present authors made a further step by testing the influences of hexoses other than D-glucose and other carbohydrates as well as glycerol and DL-lactate on the reaction, with such results as are given in Table II.

TABLE II

Effects of Carbohydrates and Related Substances on Enzymic Degradation of D-Glucosamine by Washed Suspensions of Esch. coli K_{12}

Substance added	Final concentration	Substrate		
		D-Glucosamine		Substance added
		$Q_{NH_3}(N)$	$Q_{O_2}(N)$*	$Q_{O_2}(N)$
None		296	526	—
D-Glucose	M/50	13	446	484
〃	M/100	64	415	572
〃	M/200	213	467	348
D-Fructose	M/200	256	525	396
D-Mannose	M/200	144	544	355
D-Galactose	M/200	293	517	68
Maltose	M/200	293	541	39
Lactose	M/200	294	535	30
L-Arabinose	M/200	286	514	26
D-Xylose	M/200	310	542	52
Glycerol	M/200	294	556	48
Sodium DL-lactate	M/200	307	546	35

* This $Q_{O_2}(N)$ value corresponds to $Q_{O_2}(N)$(glucosamine+substance added).

With respect to deamination of glucosamine, the higher the concentration of glucose, the stronger the inhibitory effect; the addition of glucose in $M/50$ caused an almost complete inhibition. Of ten substances added in equal concentration of $M/200$, only three zymohexoses had inhibitory effect, the intensity of inhibition falling in the following order: D-mannose$>$D-glucose$>$D-fructose. Substances other than these three zymohexoses did neither consume oxygen substantially nor influence oxidation of glucosamine. On the contrary, fructose or mannose was oxidized at the same rate as glucose, and $Q_{O_2}(N)$ in the presence of both glucosamine and zymohexose was found much smaller than the sum of $Q_{O_2}(N)$ in the presence of glucosamine alone and $Q_{O_2}(N)$ in the presence of zymohexose alone. As will be seen from values of $Q_{O_2}(N)$ (glucose) in Table II, the enzymic oxidation reaction seems to exert its maximal velocity already at $M/100$ concentration of the substrate. Thus, provided that enzyme system respansible for oxidation of glucosamine (or its deaminated product) is the same with that responsible for glucose (or other zymohexose), it will be natural for the following relation to be established in higher concentrations of substrate:

$$Q_{O_2}(N)\left(\begin{matrix}\text{glucosamine}+\\ \text{zymohexose}\end{matrix}\right)<Q_{O_2}(N)(\text{glucosamine})+Q_{O_2}(N)(\text{zymohexose}).$$

And the magnitude of difference between right side and left side of the above inequality can hardly be interpreted as the quantitative expression of degree of inhibition.

Anaerobic Breakdown of Glucosamine—Anaerobic deamination of glucosamine was reported by both Lutwak-Mann (*2*) and Rosenberg (*3*). Using Warburg apparatus instead of Thunberg technique employed by precedent investigators, the authors studied anaerobic decomposition of glucosamine. As shown in Table III, it was found that ammonia is formed in the same amount as it is under aerobic conditions, and that during the course of reaction gas pressure continues to increase steadily. This increase in gas pressure would be attributed to hydrogen production, for all of carbon dioxide liberated could be caught by the center-well mixture under the experimental conditions used. The amount of gas liberated is tentatively expressed by $Q_{H_2}(N)$ value in the tables, though the extra gas produced was not yet identified as hydrogen. The gas production occurred also when using glucose as substrate, $Q_{H_2}(N)$ (glucose) being about twice as large as $Q_{H_2}(N)$(glucosamine).

Stephenson and Stickland (*12*) confirmed that *Bact. coli* grown in broth medium containing glucose possessed formic hydrogenlyase and evolved molecular hydrogen also from glucose, fructose and mannose. Since sodium fluoride, carbon monoxide, *etc.* have been known as inhibitors of formic hydrogenlyase, the authors examined these substances for the effects on anaerobic deamination and gas production (Table III). Under anaerobic conditions sodium fluoride inhibited both deamination and gas production by about 40 per cent, though aerobically this agent did not influence the reaction so drastically. To test the effect of carbon monoxide, the air in gas phase of Warburg flask was thoroughly replaced by carbon monoxide. Hydrogen production from glucosamine as well as that from glucose was markedly depressed by carbon monoxide, whilst deamination remained intact. The addition of glucose in $M/100$ concentration resulted in complete inhibition of deamination under anaerobic condi-

TABLE III

Anaerobic Degradaiion of D-*Glucosamine by Washed Suspensions of Esch. coli* K_{12}
(Conditions as in "Standard method," except for applying anaerobic condions)

Gas phase	Addition		Substrate		
			D-Glucosamine		D-Glucose
			$Q_{NH_3}(N)$	$Q_{H_2}(N)$	$Q_{H_2}(N)$
N_2	None		296	530	1160
〃	Sodium fluoride	(*M*/100)*	198	310	972
〃	D-Glucose	(*M*/100)	5	1110†	1160
CO	None		323	65	89

* Final coflcentration.

† This value corresponds to $Q_{H_2}(N)$(glucosamine+glucose).

tions, as *M*/50 glucose did under aerobic.

Effect of Oxygen Tension—In connection with the preceding findings (Table III) that glucosamine was anaerobically deaminated with production of hydrogen, the authors intended to investigate what kind of variation in enzymic activities be brought about by gradually changing contents of gas phase in Warburg vessels from 100 per cent oxygen to 100 per cent nitrogen, and the results obtained are are collected in Table IV. Oxygen consumption decreased step by step along with the decrease in oxygen tension from one atm. pressure to zero. It was under the condition Air : N_2 = 20 : 80 that the $Q_{O_2}(N)$ was reduced to about one half of the amount observed under standard conditions. And when the anaerobiosis came to reach the degree of Air : N_2 = 10 : 90 and more, oxygen uptake was no longer observed and there was even an upward tendency in pressure, the magnitudes of the latter being given in Table IV in term $Q_{H_2}(N)$.

Worthy of special mention is the fact that while gas pressure during reaction shifts markedly from decreasing to increasing stage accompanying the change of oxygen tension, the ammonia formation on the other hand occurs in almost same rates. Attention must be paid also to the mode of variation of $Q_{O_2}(N)$ and $Q_{H_2}(N)$ under different oxygen tension: the values for glucosamine run parallel with those for glucose.

Decay of Enzyme Activity on Standing and Its Restoration—The washed suspensions of *Esch. coli* K_{12} in NaCl solution did not lose their activity to decompose glucosamine on standing for one hour at O°, but did decay upon standing overnight in ice-box at 5°. The loss of activity was markedly diminished by suspending organism in *M*/15 phosphate buffer at pH 7.2 in place of NaCl solution. Following the example shown by Gale and Stephenson (*13*), the authors tested the effect of addition of heat-treated organism

TAALE IV

Effect of Oxygen Tension on the Enzymic Degradation of D-*Glucosamine by Washed Suspensions of Esch. coli* K_{12}

(Conditions as in "Standard method," except for gas phase)

Gas phase			Substrate				
O_2	Air	N_2	D-Glucosamine			D-Glucose	
%	%	%(v/v)	$Q_{NH_3}(N)$	$Q_{O_2}(N)$	$Q_{H_2}(N)$	$Q_{O_2}(N)$	$Q_{H_3}(N)$
100	0	—	305	509	—	518	—
50	50	—	304	548	—	632	—
—	100	—	296	526	—	587	—
—	40	60	278	477	—	481	—
—	30	70	326	412	—	388	—
—	20	80	328	292	—	294	—
—	10	90	323	—	44	—	66
—	0	100	296	—	530	—	1160

to cells in order to recover the activity lost during preservation. Heat-treated organism was prepared by suspending *Esch. coli* K_{12} in a concentration three fold of the washed suspension used in the preceding enzymic tests, and sreatiog it at 70° for 20 minutes to destroy its enzymic activity. It is clear from the Table V that heat-treated organism effects recovery of deaminating activity up to one half of the power of fresh suspension and that the active, heat-stable principle in heat-treated organism can not be substituted by ATP. ATP with magnesium ions, nevertheless, exhibited stimulatory effect upon enzymic activities of the organism either preserved in phosphate buffer or kept in NaCl solution with resultant decay of the activity which was restored by the addition of heat-treated organism. A quite similar pattern of decay and restoration of enzymic activities was found when a parallel study was made on glucose, and the data obtained are given in Table V for comparison.

DISCUSSION

It was pointed out by Lutwak-Mann (*2*) in 1941 that the enzyme system of mammalian tissues, yeast and bacteria concerned with the amino sugars seems to be composed of two closely linked enzymes, of which one is responsible for the oxidation and the other for the deamination of the substrate. From the observations that the rates of glucosamine disappearance and oxygen uptake were greater than that of ammonia formation, she presumed tyat glucosamine was first oxidized to a certain

TABLE V

Decay of D-*Glucosamine-decomposing Activity on Standing of the Washed Suspensions of Esch. coli K_{12} Overnight at 5° and Restoration of the Activity by Addition of Heat-treated Organism and ATP*

Contents of Warburg flasks: 1.2 ml. of *M*/15 phosphate buffer (pH 7.2), 0.15 ml. of *M*/10 substrate solution, 0.6 ml. of enzyme solution, and solutions of appropriate substance or water to make up total volume 3.0 ml.

Suspending medium	Addition	Substrate		
		D-Glucosamine		D-Glucose
		$Q_{NH_3}(N)$	$Q_{O_2}(N)$	$Q_{O_2}(N)$
0.9% NaCl	None	51	9	8
〃	Heat-treated organism*	147	320	459
〃	ATP (*M*/200)† $MgCl_2$ (*M*/1000)	42	18	11
〃	Heat-treated organism ATP (*M*/200) $MgCl_2$ (*M*/1000)	260	494	551
M/5 Phosphate buffer (pH 7.2)	None	189	336	439
〃	ATP (*M*/200) $MgCl_2$ (*M*/1000)	236	441	537

* 0.3 ml. of heat-treated organism prepared by treating bacterial suspension (3 mg. N/ml.) at 70°C for 20 minutes was used for each experiment.

† Final concentration.

acid substance and the latter then deaminated. Some of the results obtained by herself, however, might suggest that the preceding process be the deamination rather than the oxidation: namely, the strong inhibition of oxidation by potassium cyanide or glyceraldehyde without much affecting the deamination; and anaerobic deamination bp bacteria even in the absence of hydrogen acceptor like methylene blue. In fact, Rogers (*4*) established that the rates of N-acetylglucosamine utilization, deamination and lactate formation by streptococci were equal, and the present authors, moreover, observed that in the course of D-glucosamine degradation by *Esch. coli* amount of ammonia formation reached its maximum before oxygen consumption did the same, as shown in Fig. 1; all of these are not favourable to the hypothesis presented by Lutwak-Mann that oxidation precedes deamination.

Here the authors regard following three findings as important. First of all, inhibitors of respiration such as cyanide, arsenite, and fluosilicate caused strong depression of oxidation in such low concentrations that no effect deamination was observed (Table I). Secondly, the deamination occurred with almost consistent rate under any set of condition whatever the gas phase, be it strictly aerobic or strictly anaerobic (Table IV). And finally, under anaerobic condition, the deamination was not affected by carbon monoxide while hydrogen production was inhibited strikingly (Table III). These findings have led the authors to a presumption that, whatsoever the condition of gas phase may be, the deamination occurs prior to the oxidation and the deaminated product is in the next place decomposed with consequent oxygen consumption or hydrogen production. The authors consider that inhibitors mentioned above exert their effects only on the second step of the reaction.

Provided that glucosamine is first deaminated, what is the mode of deamination reaction: hydrolytic or dehydrogenating?—and consequently, what kind of substance is produced by deamination? The authors have not yet had enough evidence to answer these questions. However, it is possible to deduce that the product of deamination reaction may be one of zymohexoses or one of the intermediary metabolites closely related to zymohexoses, from the fact that inhibitory effect on deamination was produced only by adding any one of three zymohexoses among carbohydrates tested (Table II). Similar findings were obtained also with glucose and glyceraldehyde by Lutwak-Mann and with glucose by Rogers. That $Qo_2(N)$(glucosamine) and $Qo_2(N)$ (glucose) ran almost parallel with each other under various test conditions (Tables I, II and IV) suggests the "overlapping" of two enzyme systems, the one responsible for oxidation of glucosamine and the other for that of glucose. As regards the overlapping point, the authors are of opinion that glucosamine is first deaminated to form a zymohexose-like substance and the latter is next oxidized through the same enzyme system that oxidizes zymohexose itself.

According to Stephenson and Stickland (*12*), the substrate from which appreciable amounts of hydrogen are produced by the action of *Bact. coli* has been limitted to formic acid and three zymohexoses, and the present authors have herewith added D-glucosamine to the list of substrate for hydrogen production. Here again the authors' view, that glucosamine is first deaminated and then decomposed in the same way as holding for zymohexose, accounts well for the mechanism of hydrogen production from glucosamine. That glucose exhibited inhibitory effect on deamination also under anaerobic condition, and that the inhibition of hydrogen production by carbon monoxide occurred to the same extent both for glucosamine and for glucose are the additional facts that give support to the views of the present authors as to the mechanism of reaction.

In concluding above discussions, the authors are of opinion that D-glucosamine is deaminated prior to oxidation, the deaminated product is a zymohexose-like substance, and the latter undergoes oxidation aerobically, or decomposition down to hydrogen production anaerobically as zymohexose does. Here is left a problem of importance. It is to isolate and identify the zymohexose-like substance, which is a probable intermediary metabolite derived from glucosamine.

Arsenite under aerobic conditions (Table I) and fluoride under anaerobic (Table III) exerted smaller effect on glucose than on glucosamine in term of $Q_{O_2}(N)$ and $Q_{H_2}(N)$, respectively. The exceptional results of this kind with fluoride can be explained when it is assumed that this agent attacks deaminating step in anaerobic condition, but the authors have failed to elucidate the reason why arsenite inhibits oxidation of glucose more strongly than it does that of glucosamine, in spite of the fact that deamination remains almost intact in this case.

The authors' observations that washed suspensions of *Esch. coli* in NaCl solution decayed on standing overnight in their enzymic activity to decompose glucosamine, and that activity was restored almost completely by adding heat-treated organism and ATP are of interest, for they help clarify the mechanism of glucosamine decomposition. Although it has been established that the active principle in heat-treated organism is not ATP, its actual nature remains still obscure. Gale and Stephenson (*13*) observed that suspensions in distilled water of *Bact. coli* lost 60—80 per cent of their deaminase activity toward serine on standing for 3 hours at 37°, and that the decay could be prevented by suspending bacteria in *M*/100 phosphate buffer or recovered by adding boiled organism, glutathione, formate and others. This kind of resemblance between the pattern of decay and restoration of enzymic activity concerning glucosamine as observed by the present authors and the findings concerning serine deaminase reported by Gale and Stephenson encourages the authors to make further investigation in this direction.

SUMMARY

1. Washed suspensions of *Escherichia coli* K_{12} decompose D-glucosamine to form ammonia, with consequent oxygen consumption aerobically or gas (probably hydrogen) production anaerobically.

2. The rate at which oxygen is taken up in slower than that of ammonia formation. The enzyme is almost equally active over the range of pH 6.5—8.0.

3. All of the inhibitors of respiration such as cyanide, arsenite, and fluosilicate depress ttrongly oxidation rather than deamination of D-glucosamine, and under anaerobic conditions carbon monoxide inhibits hydrogen production markedly, while deamination remains unaffected. Almost all of agents tested as well as oxygen tension in gas phase affect the oxidation of or hydrogen production from both D-glucose and D-glucosamine in the same way.

4. The addition of D-glucose to the reaction mixture causes marked inhibition of D-glucosamine deamination, either aerobically or anaerobically, and this kind of inhibition is observed also with D-mannose or D-fructose, but not with other carbohydrates, glycerol, or DL-lactate. The inhibitory effects of zymohexoses fall in the following order: D-mannose>D-glucose>D-fructose.

5. Washed suspensions lose their activity towards D-glucosamine on standing in NaCl solution overnight at 5°, the loss of activity being partly prevented by suspending bacteria in phosphate buffer. The cells that already decayed are reactivated by adding heat-treated organism.

6. Discussions are made on the mechanism of D-glucosamine degradation, and the data obtained are interpreted from the authors' point of view that D-glucosamine is deaminated to a zymohexose-like substance prior to oxidation and then the deaminated product ungergoes degradation to the final through the same enzyme system with that acting on zymohexose.

The authors wish to express their deep gratitude to Professor Senji Utzino, M. D., Kyoto University, for constant guidance given during the course of this work.

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ENZYMIC DEGRADATION OF D-GLUCOSAMINE BY ESCHERICHIA COLI

II. VARIATION OF D-GLUCOSAMINE DEAMINASE ACTIVITY DUE TO CONDITIONS DURING GROWTH

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During the course of an investigation on the *coli*-enzyme which decomposes D-glucosamine (*1*), it has become urgent for the authors to obtain organism possessing as high activity as possible. For this purpose the authors have tested the effect of the addition of glucose to the culture medium and found it to produce a stimulating effect on the formation of enzyme to deaminate glucosamine. The present paper deals with this stimulation in some detail. As reported previously (*1*), the deamination occurs prior to oxidation in the glucosamine decomposition. In this communication, therefore, interest has centered on the deaminase activity of the organism grown under various conditions. The data for oxygen consumption have been added for reference.

Gale (*2*) reviewed the variations in detail of such enzyme activities of microorganisms, as succinic dehydrogenase, formic hydrogenlyase, glucozymase, *etc.*, due to the various conditions during bacterial growth. As regards enzyme concerned with decomposition of glucosamine, however, discussions of this kind have been made neither by Gale nor by any one of precedent investigators (*3—5*).

EXPERIMENTAL

Bacteriological—Organisms used in this study were *Escherichia coli* K_{12}, B_2, and No. 1, all of which were obtained from the Research Institute for Microbial Diseases, Osaka University, and maintained on broth agar slant before inoculation. The basal culture medium was composed of 3 per cent peptone and 0.5 per cent NaCl solution, its initial pH being 5.8. If necessary, the pH of the media was adjusted with 1 *N* HCl or 1 *N* NaOH before sterilization. The medium was sterilized by autoclaving under 7 lbs, pressure for 10 minutes. For comparative study of effects of different pH or substances added to the medium, the culture was sterilized by filtration through Toyo filter paper No. 85, any drastic sterilization procedure being avoided. Bacteria were cultivated in 200-300 ml. of medium placed in 500-ml. Erlenmeyer flask at 37° for 18 hours without agitation.

Enzyme Preparation—Washed suspensions of bacteria in 0.9 per cent NaCl solution, prepared in such a way as previously reported (*1*), contained about 1 mg. bacterial nitrogen per ml. (*6*).

Measurement of Enzyme Activities—The amount of ammonia formed and oxygen consumed was determined by Conway's microdiffusion method and the Warburg-manometric method, respectively, under the same conditions with those described in "Standard method" in the preceding paper (*1*). The enzymic activities were expressed in terms of Q values as before (*1*), their definitions and mean values for *Esch. coli* K_{12} grown in the presence of 2 per cent glucose being as follows:

$Q_{NH_3}(N)$=μl. NH_3 formed per mg. bacterial nitrogen per hour, when D-glucosamine is used as the substrate.

Mean value (9 cases)=331±8.2*.

$Q_{O_2}(N)$=μl. O_2 taken up per mg. bacterial nitrogen per hour, when D-glucosamine is used as the substrate.

Mean value (9 cases)=571±16.8*.

The values of enzyme activity are shown without correcting for those obtained in blank tests, since the latter were negligible as in the preceding study (*1*).

RESULTS

Enzyme Activites of Three Strains of Esch. coli—It held for any one of the three strains of Esch. coli tested that the organism grown in the presence of D-glucose showed higher activity of glucosamine deamination than that grown in the absence of glucose (Table I). The following experiments were carried out with the K_{12} strain, the deaminase activity of which seemed to be most markedly stimulated by the addition of glucose among the three.

TABLE I

D-*Glucosamine-decomposing Activities of Esch. coli Grown in Media with and without Glucose*

Strain	D-Glucose (2%)	Final pH	$Q_{NH_3}(N)$	$Q_{O_2}(N)$
Esch. coli K_{12}	None	7.6	197	754
	Added	4.8	331	571
Esch. coli B	None	6.8	155	680
	Added	4.8	245	546
Esch. coli No. 1	None	7.6	198	739
	Added	4.8	318	466

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* Standard error of the mean.

Effect of Substances Added to Culture Medium—Variations of deaminase activity due to the addition of sugars other than glucose, glycerol, and sodium lactate were studied besides glucosamine, the substrate itself. Table II shows that stimulation of the activi- ,y occurs to almost same extent by adding D-glucosamine, hexoses, and disaccharides to the growth medium. Particularly, the presence of D-mannose and D-galactose in the medium was more effective on activity than that of glucose. On the contrary, in the case of pentose such as L-arabinose and D-xylose, the increase in activity was not so marked as in the case of hexoses, and the addition of glycerol and DL-lactate exerted non-simulatory or rather inhibitory effect on the deaminase activity.

TABLE II

Effect of Culture Media on the D-*Glucosamine-decomposing Activity of Esch. coli* K_{12}

Substance added*	Final pH	$Q_{NH_3}(N)$	$Q_{O_2}(N)$
None (control)	7.6	197	754
D-Glucosamine	5.0	346	998
D-Glucose	4.8	331	571
D-Fructose	4.9	322	516
D-Mannose	5.0	435	681
D-Galactose	4.9	371	654
Maltose	5.0	317	584
Lactose	4.8	324	531
L-Arabinose	4.8	211	458
D-Xylose	4.8	250	506
Glycerol	5.8	203	711
Sodium DL-lactate	7.2	106	562

* One per cent in all cases excepting the case of glucose (two per cent).

Effect of Concentration of Glucose Added to Culture Medium—As shown in Fig. 1, the stimulation of activity by glucose can be effectuated even at its level of 0.1 per cent, and gradual increase occurs till the maximum stimulation is obtained at 2 per cent glucose.

Successive Cultivation—Fig. 2 shows that a single cultivation in the presence of glucose is sufficient to activate the deaminase to its full extent, and a single subsequent cultivation in the absence of glucose results in the decrease of the enzyme. This clearly indicates the fact that the stimulation of activity by adding glucose is due to the temporary change of the enzyme formation.

Effect of Medium pH—The nature of stimulation by glucose is, however, still obscure: the problem is whether glucose produces this effect directly by acting upon the enzyme or its production, or whether it is due to the action of some less specific metabolites

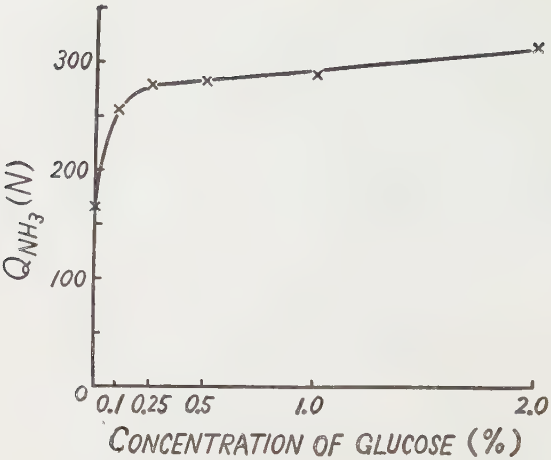

FIG. 1. Effect of D-glucose concentration on the D-glucosamine deaminase activity of *Esch. coli* K_{12}.

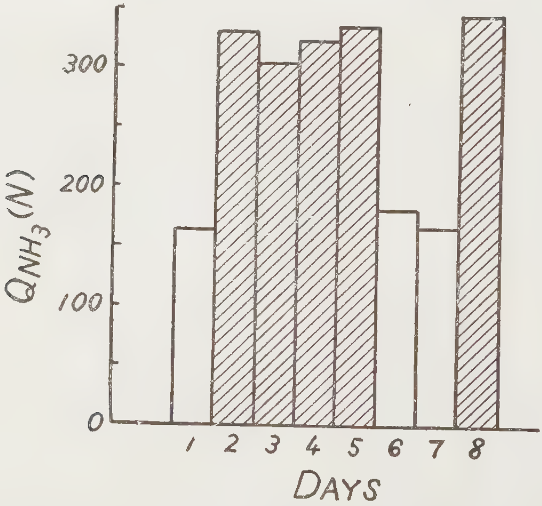

FIG. 2. Variation of D-glucosamine deaminase activity due to successive cultivation: *Esch. coli* K_{12}.

Successive cultivation was made in daily turn as shown above, the basal medium (clear column) or the medium containing 2 per cent D-glucose (cross-hatched column) being used, brought about by the presence of glucose during bacterial growth.

In most cases where glucose is metabelized, its presence in the growth medium leads to an alteration of the environmental pH. Gale (*2*) found that the addition of glucose had an stimulatory effect on hydrogenase, catalase and arginine and lysine decarboxylases activity of *Esch. coli*, and established that this effect of glucose was due to the change of the acidity during fermentation, as organisms grown in the basal medium receiving previous acidification were as active as cells grown in the presence of glucose. As indicated in Table III, it occurs in all cases, except one with phthalate buffer, that the final pH of the media shifts toward neutral or alkaline side by 0.8-1.8 from the different initial values. The organisms grown in these media showed no higher activity than organism grown in the basal medium (control). Further, even in case the shift of the medium pH during growth was minimized by the addition of phthalate buffer (pH 4.8), no remarkable stimulation of activity resulted. These findings indicate that the stimulation of glucosamine deaminase activity by the addition of glucose to the growth medium is not due to the effect of the acidity resulting fɹom fermentation of glucose.

TABLE III

Variation of D-*Glucosamine-decomposing Activity Due to pH of Basal Medium during Growth: Esch. coli* K_{12}

Intial pH	Final pH	$Q_{NH_3}(N)$	$Q_{O_2}(N)$
5.8 (control)	7.6	197	754
4.5	6.0	182	825
4.7	6.0	190	768
4.8*	5.2	209	783
5.0	6.6	192	682
5.5	6.8	189	744
7.0	7.8	184	744

* Phthalate buffer added (final concentration *M*/60).

Effect of Oxygen Tension During Growth—Since gas is produced by fermentation of glucose during bacterial growth and it gives rise to anaerobiosis, the authors intended to study the effect of oxygen tension during growth on the variation of glucosamine deaminase. The variation of oxygen tension due to the gas forming in the medium might be reflected to the variation of enzyme activity. In fact, Stephenson and Gale (*7*) demonstrated the difference in deaminase activity towards glycine and alanine between *coli* organisms grown aerobically and anaerobically.

As shown in Table IV, in case the aerobic conditions of growth are brought about either by aeration during cultivation or by surface culture on agar, the glucose added exhibits no longer stimulatory effect on glucosamine deaminase, though pH of the

TABLE IV

Variation of D-*Glucosamine Deaminase Activity Due to Oxygen Tension during Growth : Esch. coli* K_{12}

Condition	D-Glucose (2%)	Final pH	$Q_{NH_3}(N)$	$Q_{O_2}(N)$
Aerobic (Aeration)*	None Added	8.2 4.7	173 141	734 633
Aerobic (Agar culture)†	None Added	8.0 4.8	147 155	542 513
Anaerobic (N_2)	None Added	6.2 4.8	352 328	660 450

* Bubbling the culture with air stream throughout the cultivation period.

† In Roux bottle, 3 per cent agar.

medium falls 4.7–4.8 by glucose fermentation. This observation suggests that the stimulatory effect of glucose is closely related to the fermentation gas formed. To confirm this view, cultivation was made under 1 atm. pressure of nitrogen, and by doing so it was possible to increase the enzymic activity of organism grown in the absence of glucose to the same level with that of organism grown in the presence of glucose.

Effect of the Age of Culture—In may microorganisms, the maximal activity of their enzymes is usually shown when the maximal growth has been attained, and Fig. 3 shows that glucosamine-decomposing enzyme of *Esch. coli* K_{12} is not exceptional. The same figure includes the growth curve of bacteria as plotted by the turbidity of the medium measured photoelectrically, together with the alteration curve of the medium pH as measured by glass electrode. The increase in turbidity began at the 3rd hour of cultivation, and continued till the 10th hour logarithmically to time. The enzyme seems to be formed during this period, and its activity to deaminate glucosamine was considerably high already at the 6th hour. It evoked its full activity between the 14th and the 20th hour, when turbidity was stabilized, followed by a slight decrease. On the other hand, oxidizing activity reached its highest level betwee the 10th and the 14th hour—earlier than deaminating activity reached the peak. The pH of the medium during growth began to decrease simultaneously with the beginning of logarithmic phase of the growth and kept falling gradually until the turbidity became stabilized at the 14th-16th hour.

DISCUSSION

It has been observed with *Esch. coli* K_{12} that the addition of D-glucose to the growth medium stimulates remarkably (about 1.8 fold) the formation of enzyme to deaminate D-glucosamine (Table I). Glucose fermented may bring about some changes to the environment of growing bacteria ; hence to their enzyme constitution. Now as for the effect of

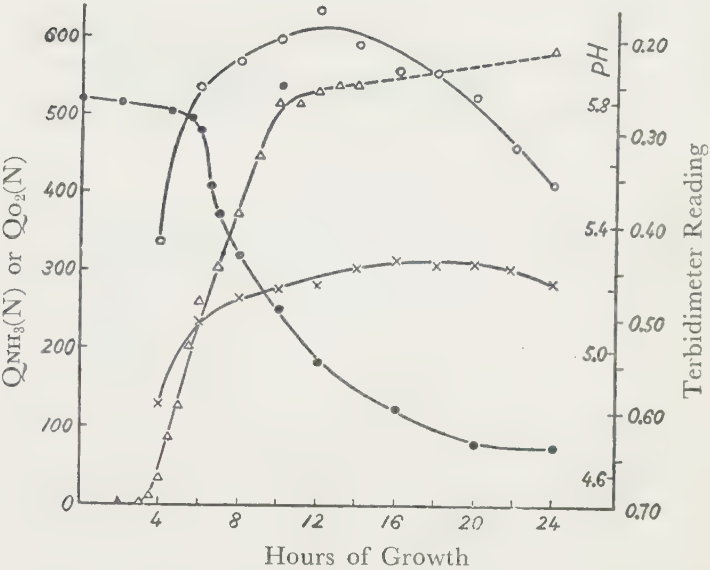

Fig. 3. Effect of the age of culture on the D-glucosamine-decomposing activity of *Esch. coli* K_{12}.
×—× Q_{NH_3}(N), ○—○ Q_{O_2}(N), △—△ Turbidimeter reading, ●—● pH of the medium.

acid formation, it is clear from the following facts that this stimulatory effect of glucose is not due to the acidity caused by its fermentation: 1) When the addition of glucose was omitted, no stimulation of deaminase activity could occur even when the growth medium was adjusted to acid pH (Table III). 2) When adding sugars such as arabinose and xylose (Table II), no marked stimulation of activity was obtained despite the fact that pH fell to 4.8 like in the case of adding glucose. 3) No stimulatory effect was manifested when cells were cultivated under aeration or on agar, though the glucose present in the medium shifted the pH towards acidity as in usual culture (Table IV).

As to the effect of gas produced by fermentation, there are such findings (Table IV) that the removal of gas from the medium results in disappearance of stimulatory effect, while, conversely, anaerobic cultivation gives rise to stimulation even in the absence of glucose. It follows that the stimulatory effect is due to the anaerobiosis brought about by the gas formation from glucose fermented during growth.

It is of much interest to see the fact that the formation of enzyme to deaminate glucosamine is clearly stimulated by adding to the medium hexoses or disaccharides which give hexoses on hydrolysis, whereas the very hexoses, when added to the reaction mixtures with washed *coli* suspension, inhibit the enzymic reaction to deaminate glucosamine as reported previously (*1*).

There is an additional point worthy of attention. As shown in Tables I-IV, contrary to the increased ammonia formation, the oxygen consumption due to the degradation of glucosamine is less for bacteria grown in the medium containing glucose than for those grown in the basal medium. The same sort of phenomena was encountered during the experiments where glucose was used as substarte under the same conditions with those instituted when using glucosamine as substrate. Stephenson and Gale (*8*) have already demonstrated that glucozymase of *Bact. coli* is remarkably strengthened in its activity by the addition of glucose to the growth medium. When putting thi findings of theirs together with those of the authors', it can be understood that glucose is decomposed more rapidly by organisms grown in the presence of glucose than by those grown in the absence of glucose, while on the contrary, the oxygen consumption per one mole of glucose utilized is less for the former than for the latter. In other words, with bacteria grown under different conditions, the magnitudes of $Q_{O_2}(N)$ are not always proportional to the enzymic activities to decompose glucose or glucosamine.

SUMMARY

1. D-Glucosamine deaminase activity of *Escherichia coli* K_{12} is markedly stimulated when the cells are grown in the medium containing D-glucose. It is concluded that this stimulatory effect is not due to the acidity brought about by fementation of glucose, but due to the anaerobiosis owing to fermentation gas formed during growth.

2. The degrees of stimulation are observed to be almost invariable for any level of the concentration of glucose between 0.25 and 2.0 per cent. The stimulation of the enzyme activity appears to be a temporary change in the organism.

3. The organism grown in the presence of glucose shows the highest activity of glucosamine deaminase when harvested at the time of its maximal growth (around the 16th hour of cultivation).

4. The presence of D-glucosamine, hexoses other than glucose,

and disaccharides in the culture medium also exhibits stimulatory effect on the D-glucosamine deaminase activity.

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RECHERCHES SUR LA FORMOL-GÉLIFICATION DES PROTÉINES

I. CARACTÉRISTIQUES DE L'ALBUMINE ET DE L'EUGLOBULINE AU POINT DE VUE DE LA CONCENTRATION DU FORMOL Y AJOUTÉ.*

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INTRODUCTION

On a beaucoup discuté, depuis longtemps et dans de nombreux domaines, des réactions de la protéine contre le formol. Il est bien connu que le formol est un réactif très employé, dans le domaine de la chimie de protéine, pour le titrage du formol de Sörensen,, et il est aussi nécessaire, dans le domaine de l'immunologie, pour fabriquer des toxides d'après Ramon.

Mais ce qui nous intéresse, c'est un phénomène physico-chimique que la solution de protéine (sérum) se prend en gel en présence du formol. Depuis qu'en 1920 Gaté et Papacostas (*1*) avaient découvert, par hasard, ce phénomène, beaucoup de savants comme Napier (*2*), Nicolas (*3*), Kürten (*4*), Napier-Lloyd et Paul (*5*), Chopra et Choudhury (*6–8*), Biguria et Foster (*9*), Vershure (*10*, *11*), Milhaud (*12*) *etc.* ont fait des études plus poussées concernant cette réaction, à savoir : sur le rapport entre cette réaction et diverses maladies ; sur la composition de la protéine de sérum réactive au formol; sur la measure de la concentration de la globuline contenue dans le sérum en profitant de cette réaction. Nous croyons que le mécanisme de la formol-gélification consiste en des métamorphoses chimiques ou physico-chimiques, mais la plupart des études ont été faites au point de vue clinique, Bien sûr, on a discuté vivement dans l'étude physico-chimique de la protéine, les réactions basées sur la combinaison du formol avec l'amino-acide, la polypeptide, la protéine, etc. Malgré cela ce n'est que A. B. Gutman (*13*) pui s'set intéressé au mécanisme de formol-gélification par suite de ses recherches sur les dites réactions. Même lui n'a traité que brièvement l'influence de "CH_2-liaison" sur la formol-gélification dans son article concernant la plasma-protéine.

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* Le sommaire de ce mémoire a été presenté au 24e Congrès de Biochmie du Japon, Symposium de la plasma-protéine, le 4 Mai, 1952.

D'autre part, dans notre pays Abiko (*14*), Yamaguchi (*15*) Komano (*16*); *etc.* ont rendu compte de cette réaction. A la suite de ces recherches, Suzuki (*17–21*), suivant l'indication de Prof. Miyamoto, a observé quantitativement comment le sérum de Kala-azar préalablement fixé à divers pH réagit en présence du formol, en mesurant la viscosité de ce sérum qui change selon le temps écoulé après l'addition du formol. Et il a découvert divers faits nouveaux. Le résultat de ses observations nous fait apprendre que le pH a une influence importante sur la viscosité du sérum. Elle change comme suit lorsqu'on ajoute du formol. Primo : la viscosité augmente du côté de l'acidité dès le pH 5,4; Secundo : elle s'élève brusquement dans les limites extrêmement étroites auprès du pH 7 au pH 8; Tertio : elle monte aussi du côté de l'alcalinité (au-dessus du pH 9,6). Le premier et le troisième phénomènes sont visibles soit puand on ajoute du formol au sérum, soit qu'on n'en ajoute pas. Par conséquent, il semble qu'on doive les considérer respectivement comme gélification par l'acide fort et celle par l'alcali fort n'ayant aucun lien avec la formol-gélification. Le deuxième phénomène, au contraire, n'a lieu pu'en présence du formol. Suzuki a révélé que le lacto-gel, quoiqu'il soit un de divers acide-gels, est tout à fait différent d'un gel causé par l'acide minéral fort, au point de vue du pH auquel se produit une gélification du sérum.

Nous nous intéressons à la méthode de Miyamoto et Suzuki qui suivent le changement de la viscosité jusqu'à ce qu'il apparaisse un gel et ainsi traitent quantitativement le problème de gélification. En même temps nous voulons étudier la formol-gélification en profitant des récentes connaissances physico-chimiques sur la protéine. Pour ce but, nous avons extrait du sérum de cheval de l'euglobuline, des pseudoglobuline I et II et de l'albumine. Et nous avons observé comment ces fractions réagissent contre le formol, en suivant le changement de leur viscosité à divers pH. Comme nous avons déjà dit, le but de notre précédent rapport (*22*, *23*) était de suivre de quelle façon réagit en présence du formol chaque fraction protidique de sérum. Dans ce rapport, nous avons constaté que, parmi les fractions protidiques contenues dans le sérum, ce n'est que l'euglobuline qui réagit remarquablement contre le formol. Le phénomène de formol-gel est la caractéristique de l'euglobuline dont la viscosité atteint le point le plus haut entre le pH 6 et le pH 9, surtout dans les limites extrêmement étroites du pH 7 au pH 8.

Dans ce cas-là, nous avons fait notre expérience en ajoutant une goutte de formol à 1 ml. de la solution de protéine. Mais, comme des réactions chimiques sont, en général, gravement influencées par la concentration des réactifs, il nous semble très intéressant d'étudier de quelle manière l'euglobuline réagit et se transforme si l'on y ajoute du formol de différentes concentrations. En ce pui concerne l'albumine qu'on croyait qu'elle reagissait peu contre le formol dans la condition habituelle de formol-gélification, nous avons aperçu de temps en temps au cours de nos expériences que cette fraction aussi pourrait changer sa viscosité auprès du pH 4.8. De là, nous arrangerons à plusieurs pH les solutions également concentrées d'euglobuline et d'albumine avec du formol de différentes concentrations, et suivrons comment peut saltérer leur viscosité.

PARTIE EXPÉRIMENTALE

Préparation et composition Électrophorétique des Fractions Protidiques—Pour la présente expérience, nous employons l'euglobuline et la sérumalbumine A de cheval. Elles sont relargagnées du plasma de cheval, raffinées et recristallisées trois fois en utilisant Na_2SO_4 en poudre (*24–27*). Aprés les avoir dialysées sous pression et leur enlevé du sel, nous leur ajoutons du chlorure de sodium à 0,9 p. 100. Nous mesurons N dans les solutions filtrées de ces fractions protidiques selon la méthode de Mikro-Kjeldahl pour déterminer la concentration de la protéine. Ensuite nous les employons pour nos expériences.

Ces deux fractions sont assez purifiées. Leur composition électrophorétique est comme suit: L'euglobuline continent à peu près 85 p. 100 de γ-globuline. Le reste est composé de diverses fractions, surtout de β-globuline. Cela est constaté au moyen de l'analyse gravimétrique des diagrammes èlectrophorétiques. Quant à l'albumine, elle contient à peu près 88 p. 100 d'albumine, le reste composant de diverses globulines, surtout α-globuline.

Mèthode d'Expèrience—Nous suivons la mèthod que Suzuki a appliquée déjà au sèrum de Kala-azar (17–21). Profitant de l'action tampon de la solution de protéine, et se sérvant de HCl et de NaOH dissous dans la solution de NaCl à 0,9 p. 100, nous prèparons à plusieurs pH une sèrie de solutions de protéine dont la concentration et la quantitè sont les mêmes. Et mesurant la viscosité de chaque solution, nous comparons graphiquement le changement de la viscosité à plusieurs pH.

Pour effectuer cette méthode, nous prèparons une série de 16 tubes à essai, chacun contenant la solution de protéine traitee d'apres Tableau I. A chaque série de ces solutions montrant 16 sortes de pH, on ajoute une goutte de formol deux fois dilué, une goutte, deux gouttes et trois gouttes de formol. Mais quant à l'euglobuline, elle réagit

TABLE I

Numéro des tubes à essai	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
/N HCl	0.15															
N/5 NCl		0.15	0.08	0.05	0.03	0.02	0.01									
N/5 NaOH									0.01	0.02	0.03	0.05	0.07	0.10	0.15	0.20
0.9% NaOH	0.35	0.35	0.42	0.45	0.47	0.48	0.49	0.50	0.49	0.48	0.47	0.45	0.43	0.40	0.35	0.30
Somme totale (ml.)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Sotution de protéine (ml.)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

tout sensiblement contre le formol. Ce fait rendra très difficile à obtenir les résultats d'experience désirés, si l'on s'attache à telle méthode que de régler la quantité du formol par le nombre de gouttes. Par conséquent, nous ajustons la concentration finale du formol respectivement à 0,16, 0,33, 0,99 (à peu près une goutte), 1,32, 1,65, 1,98, 2,64 et 3,95 p. 100, en modifiant la quantité de la solution de NaCl.

Comme le formol commercial contient de l'acide formique, on y ajoute, pour le neutraliser, du carbonate de calcium précipité. Ensuite, analysant quantitativement le formol ainsi préparé suivant le méthode de Blank-Finkenbeiner, un peu modifiée, nous l'appliquons à nos expériences. Le viscosimétre de Hess est employé pour mesurer la viscosité.

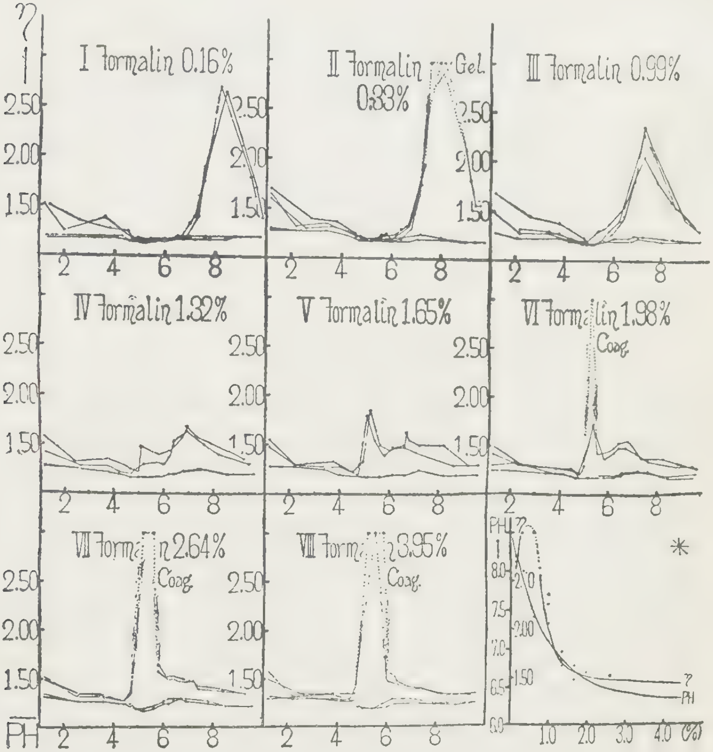
RÉSULTATS D'EXPÉRIENCE

L'influence sur la Courbe de Formol-gélification de l'Euglobuline—On arrange à une série de pH les solutions d'euglobuline ayant la concentration de 1,30 g./dl et contenant du formol de 0,16 p. 100 à 3,95 p. 100. Observant ces solutions immédiatement après et 24 heures après l'addition du formol, nous avons obtenu les résultats qu'indiquent Fig. 1 I–VIII.

Les courbes de viscosité sont plus basses immédiatement après (-×-×-) que 24 heures après (-○-○-) l'addition du formol, à divers pH aussi bien qu'à tous les degrés de concentration ci-dessus indiqués du formol. Quant aux courbes que décrit la viscosité immédiatement après l'addition du formol, on remarque une concavité auprèi des pH 5,2–5,4 en tous les cas, quelle que soit la concentration du formol. Ce phénomène est le même qu'avant l'addition du formol. Du reste, toutes ces courbes indiquent la plus haute viscosité auprès des

Fig. 1

Influence de la quantité du formol sur la courbe de formol-gélification de l'euglobuline

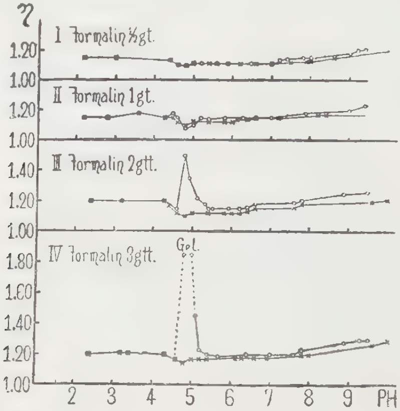


* Rapport entre la valeur maximum, la position de la convexité de la courbe de viscosité et la quantité du formol.

pH 7–8.

Au contraire, 24 heures après l'addition du formol, chaque courbe de viscosité diffère évidemment l'une de l'autre sur le compte de la hauteur et de la position d'une convexité auprès des pH 7–8 suivant la concentration du formol. C'est-à-dire, quand le formol est peu concentré (par exemple 0,16 p. 100), on voit une concavité auprès des pH 5,2–5,4 dont l'existence est constatée avant l'addition du formol. Dans ce cas on reconnait l'augmentation de la viscosité du côté de l'acidité dès les pH 5,2–5,4, et la courbe de viscosité représente une convexité toute particulière à l'euglobuline dans les limites extrêmenmet étroites auprès des pH 8–9. Cette convexité s'élève temporariement à mesure que la quantité du formol augmente, mais ensuite

Fig. 2. Influence du nombre de gouttes du formol sur la courbe de formol-gélification de l'albumine



elle s'abaisse. Lorsque la concentration du formol dépasse 1 p. 100, avec l'abaissement d'une convexité auprès des pH 7–8, une autre convexité apparît auprès des pH 5,2–5,4 et se met à èlever à mesure qu'augmente la quantité du formol. Ainsi la courbe de viscosité vient à avoir deux convexités. D'ailleurs si la quantité du formol est plus de 2,64 p. 100, la convexité auprès des pH 7–8 disparaît complétement. Par conséquènt on ne voit une convexité de viscosité et un phénomène de coagulation qu'auprès des pH 5,2–5,4.

Comme Fig. 1 montre, le plus haut point de ces courbes de viscosité auprès des pH 7–8 se déplace peu à pue vers le côté de l'acidité parallèlement au changement de sa hauteur suivant la quantité du formol ajouté.

D'autre part, nous observons seulement le trouble de la solution. Quand le formol est peu concentré (par exemple, moins que 1 p. 100), on remarque du côté de l'acidité dès le pH 5,2 le trouble de la solution provenant de la gélification par l'acide fort, et on voit auprès des pH 7–8 (dans les limites de formol-gèlification) un vif trouble opalescent Au fur et à mesure qu'augmente la quantité du fomol, on vient à reconnaître aussi un trouble blanc auprès des pH 5,2–5,4. Il devient plus vif à proportion que le formol est plus concentré. Au contraire, le trouble opalescent auprès des pH 7–8 (dans les limites de formolgélification) diminue peu à peu. En même temps, la position de ce trouble se déplace par degrés vers le côté de l'acidité et il finit par disparaître complètment.

L'influence sur la Courbe de Formol-gélification de l'Albumine—Fig. 2 I et II indiquent les courbes de viscosité immédiatment après (-×-×-) et 24 heures après (-○-○-) l'addition à 1 ml. de la solution d'albumine ayant la concentration de 1,30 g./dl. respectivement une goutte de formol deux fois dilué et une goutte de formol.

Comme nous avons mentionné dans notre rapport précédent, l'albumine, contrairement à l'euglobuline, est une fraction qui est très insensible et réagit peu contre le formol. Dans la condition ci-dessus indiquée, l'euglobuline montre déjà une réaction remarquable, tandis que l'albumine ne change pas sensiblement as viscosité à divers pH. Même si on lui ajoutẹ 2 ou 3 gouttes de formol (Fig. 2, III et IV), immédiatement après cela, sa courbe de viscosité montre la même concavité auprès du pH 4,8 que nous avons constatée avant d'ajouter le formol. Mais, 24 heures plus tard, l'albumine augmente sa viscosité et donne un gel dans les limites extrêmement étroites auprès du dit pH.

D'autre part, nous observons seulement le trouble de la solution. Aucun trouble ne se produit à divers pH, lorsqu'on ajoute une goutte de formol deux fois dilué, Quand une goutte en est ajouté, on ne remarque qu'un trouble très lèger auprès du pH 4,8. Au contraire, à mesure qu'augmentent des gouttes de fomol de 2 à, la solution se trouble fortement auprès du dit pH.

D'après les rèsultats obtenus concernant l'albumine, ces deux phénomnes, c'est-à-dire, le trouble de la solution et l'élévation de la viscosité, semblent se présenter presque parallèlement.

DISCUSSION

Ces résultats d'expérience nous amènent à conclure comme suit:

1° En présence du formol, une solution de protéine augmente sa viscosité ou se prend en gel auprès des pH 7-8.

2° Le même phénomène se révèle auprès des pH 4,8–5,4.

Le premier phénomène a des relations intimes avec la formol-gélification proprement dite, et c'est la caractéristique de l'euglobuline (γ-globuline) parmi des fractions protidiques de sérum. Au contraire, le second ne se révèle qu'en présence de formol plus concentré et dans ce cas l'euglobuline et l'albumine réagissent également.

Augmentation de la Viscosite ou Apparition de Gel auprès des pH 7–8—Ce qui attire notre attention, c'est le fait que pourquoi la courbe de viscosité montant le plus haut auprès des pH 7–8 s'élève encore, mais s'abaisse à l'envers à proportion qu'augmente par degrés la quantité du formol, comme indiquent les résultats d'expérience. (Fig. 1) Le formol a été considéré depuis longtemps comme un des réactifs qui dénaturent la protéine. Il y a un fait qui nous semble suggérer que ce phénomène peut avoir quelques rapports avec la dénaturation de la protéine. C'est-à-dire, d'après une observation de Tanda (*29*), la courbe de viscosité indiquant la formol-gélification de l'euglobuline dénaturée par l'alcali, est fort différente de cells de l'euglobuline non dénaturée et ressemble tout à fait à la courbe de gélification décrite lorsque le formol est très concentré. Et la convexité de la courbe de viscosité auprès des pH 7–8 diminue à mesure qu'avance la dénaturation par l'alcali et en même temps la viscosité augmente peu à peu auprès du pH 5. De là, c'est facile à comprendre ce phénomène que nous avons remarqué, comme un cas de dénaturation. Mais, comment expliquer positivement le mécanisme de ce phénomène? C'est une question. En général il est considéré que la viscosité d'une solution de protéine augmente par suite de l'addition du formol, parce que le formol fait polymériser les molécules de protéine en formant "CH_2-Liaison" avec le radical aminé de protéine et d'autres radicaux comme tyrosine, tryptophane, histidine ou peptide-union, *etc.* (*31*). Quand la protéine est dénaturée, généralement divers radicaux dans la molécule de protéine qui réagissent au formol augmentent, ce qui altère la relation quantitative entre le formol et ces radicaux, ralentissant la polymériésation des molècules de protéine. Nous croyons que c'est ainsi que l'excès de formol abaisse la viscosité de la solution de protéine. Mais il reste encore beaucoup à étudier sur ce point.

Augmentation de la Viscosité auprès des pH 4,8–5,4.—Quand une solution de protéine a une concentration de 1,30 g/dl., sa courbe de viscosité est concave auprès des pH 4,8 5,4, immédiatement après l'addition du formol très concentré ou peu concentré, comme elle l'est avant l'addition du formol. Dans ce cas l'euglobuline et l'albumine présentent toutes les deux le même phénomène. On pourra expliquer ce phénomène par le point iso-électrique. Au contraire, lorsque le formol est très concentré, par le laps de temps la viscosité de l'euglobuline et de l'albumine augmente si bien que celle-là se coagule et celle-ci se prend en gel auprès de ces pH. Il semble que ce soit un phénomène qui se rapporte à une dénaturation de la protéine, si l'on considère les faits suivants: les résultats d'expérience ci-dessus cités de Tanda (*29*) concernant la formol-gélification de l'euglobuline dénaturée par l'alcali fort, et ceux de Sano (*30*) constatant que le sérum exposé à une douce chaleur augmente sa viscosité ou se prend en gel auprès du pH 5,0 après qu'on y ajoute du formol. Voici une question : l'euglobuline se coagule, tandis que l'albumine se prend en gel; d'ou vient cette différence? Sur le compte d'une lacto-gélification, Nishi (*29*) a observé qu'une solution qui ne contient que de l'euglobuline se coagule auprès des pH 5,2–5,4, tandis qu'une autre solution où se mêlent l'euglobuline et l'albumine se prend en gel. Donc, il est possible que l'action tampon de l'albumin soit une des causes qui approtent cette différence.

RÉSUMÉ

1. Nous avons suivi le changement de la viscosité à divers pH pour préciser la formol-gélification, et constaté que la solution de pr-

téine ne réagit contre le formol en augmentant sa viscosité qu'auprès des pH 7–8 et des pH 4,8–5,4.

2. L'augmentation de la viscosité dans les limites extrêmement étroites auprès des pH 7–8 est la caractéristique de l'euglobuline (γ-globuline) parmi des fractions protidiques. Suivant la concentration du formol, la position et la hauteur de cette augmentation se changent. Plus le formol est concentré, plus le point le plus haut de la courbe de viscosité s'élève, mais ensuite il s'abaisse et enfin disparaît tout à fait, si le formol est encore plus concentré. Et on aperçoit que le pH où se trouve le point le plus haut de la courbe de viscosité se déplace vers le côté de l'acidité.

Considérant ces phénoménes, nous proposons, donc, une hypothèse que le formol en exès empêche les molécules de protéine de polymériser.

3. Quand le formol est très concentré, on peut observer l'augmentation de la viscosité auprès des pH 4,8–5,4. L'euglobuline et l'albumine présentent toutes les deux ce phénomène, qui semble être causé de la dénaturation de la protéine.

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